The innate immune response to HSV-1: glycoprotein mediated activation of dendritic cells

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I, Adi Reske, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Herpes Simplex Virus (HSV) – 1 also known as Human Herpes Virus (HHV) – 1 is a common infectious agent of humans which can cause a wide variety of clinical outcomes, ranging from mild mucocutaneous lesions to long term morbidity and possible mortality. Viral entry into cells requires the co-ordinated action of at least four HSV-1 envelope glycoproteins: gB, gD and the heterodimer gHgL. Dendritic cells (DC) are the most potent antigen presenting cells and are likely to encounter the virus early after entry into the host. HSV-1 readily infects DC leading to a series of both morphological and functional changes, and yet the pathway induced by HSV-1 that leads to DC maturation has not been elucidated. This thesis aims to understand the role of the viral entry glycoproteins in the activation of DC and the consequent initiation of an immune response. Defining this role has important implications not only in understanding immunopathogenesis, but also in the study of HSV-1 as an immunotherapeutic vector, and in the design of an efficient HSV-1 vaccine.

Monocyte-derived DCs (MDDC) were found to recognise and respond to the complex of four essential viral glycoproteins, independent of other viral proteins or nucleic acids. MDDC recognition of these four glycoproteins leads to the upregulation of a maturation phenotype and the production of type I interferon (IFN) as well as the induction of a strongly polarised T_H1, IFN-γ dominated allogeneic T cell response. In contrast, monocyte-derived Langerhans cells (MDLC), display only partial maturation phenotype and do not produce type I IFN. Plasmacytoid DC (pDC) induce a strong type I IFN response to a viral infection, but not to the viral surface glycoproteins.

In the context of natural HSV-1 infection, these results suggest a model in which at the site of HSV-1 infection, different DC sub-populations respond differently to a viral

infection and to glycoproteins expressed on the surface of infected cells in order to provide an effective immune response.

To my wife and daughter

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BHK	Baby hamster kidney
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
C	Constant
cDC	Conventional dendritic cell
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CPM	Counts per minute
CT	Cytoplasmic tail
CTL	Cytotoxic T lymphocytes
DAI	DNA-dependent activator of IFN regulatory factors
DAPI	4'-6-Diamidino-2-phenylindole
DC	Dendritic cell
DDC	Dermal dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dsRNA	Double-strandad RNA
E	Early
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy

ER	Endoplasmic reticulum
FCS	Fetal calf serum
FN	Fibronectin
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
GFP	green fluorescent protein
gG	Glycoprotein G
gH	Glycoprotein H
gI	Glycoprotein I
gJ	Glycoprotein J
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein M
HBSS	Hank’s balanced salt solution
HCMV	Human cytomegalovirus
HHV	Human Herpes Virus
HIV	Human Immunodeficiency Virus
HR	Heptad repeat
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans

HSV	Herpes Simplex Virus
HVEM	Herpes virus entry mediator
ICP	Infected cell protein
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LC	Langerhans cell
LN	Lymph nodes
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MAVS	Mitochondrial anti-viral signaling protein
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation-associated protein 5
MDDC	Monocyte-derived dendritic cell
MDLC	Monocyte-derived Langerhans cell
MMTV	Murine mammary tumour virus
MyD88	Myeloid differentiation factor 88
NF-kB	Nuclear factor-kappa B
NK	Natural killer
ODN	Oligodeoxynucleotides
PAMP	Pathogen associated molecular pattern

PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
pfu	Plaque forming units
PILR	Paired immunoglobulin-like type 2 receptor
PKR	Protein kinase R
Poly I:C	Polyriboinosinic polyribocytidylic acid
PRR	Pattern recognition receptors
RIGI	Retinoic acid-inducible gene I
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT	Room temperature
SEM	Standard error of the mean
SSC	Side scatter
SU	Surface
TAP	Transporter associated with antigen processing
TBS	Tris buffered saline
TGF	Transforming growth factor
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF	TNFR-associated factor
UL	Unique long
US	Unique short
UV	Ultra-violet

V	Variable
vhs	Virion host shutoff
VSV	Vesicular stromatitis virus
VZV	Varicella zoster virus

CHAPTER 1 - INTRODUCTION

There are eight members of the *Herpesviridae* family known to infect humans. These are classified into three subfamilies: alpha, beta and gamma (Table 1.1). All share a common structure, composed of a large double-stranded linear DNA within an icosahedral capsid wrapped in a lipid bilayer membrane.

Alpha	Herpes Simplex Virus 1 (HSV 1) Herpes Simplex Virus 2 (HSV 2) Varicella zoster virus (VZV)	Human Herpes Virus 1 Human Herpes Virus 2 Human Herpes Virus 3
Beta	Cytomegalovirus (CMV) Roseolovirus	Human Herpes Virus 5 Human Herpes Virus 6 Human Herpes Virus 7
Gamma	Epstein-Barr Virus (EBV) Kaposi's sarcoma-associated herpesvirus (KSHV)	Human Herpes Virus 4 Human Herpes Virus 8

Table 1.1: The subfamilies of the *Herpesviridae* family

A herpesvirus infection begins with attachment to and penetration of a host cell, followed by viral DNA migration to the host's nucleus where the immediate-early genes are expressed even in the presence of inhibitors of protein synthesis. The immediate-early genes are for the most part regulatory, switching on the expression of the early genes, which are made before DNA replication. The proteins made by the expression of the early genes include enzymes involved in DNA metabolism and replication. The late genes are expressed after DNA replication, and are encoded to make structural proteins including elements of the tegument.

1.1. Pathogenesis of Herpes Simplex Virus – 1 infection

Herpes Simplex Virus (HSV) – 1/2 also known as the Human Herpes Virus (HHV) – 1/2 are two species of the herpes virus family. These viruses are able to infect humans of all ages, and it is estimated that 50% of the adult population in the UK are carriers of HSV-1, and 10% are carriers of HSV-2. The rates are much higher in other countries (UK Herpes Virus Association, accessed Sept. 2008). Infection results in a wide variety of clinical manifestations. The most common form of disease in humans are mucocutaneous lesions, which occur usually in or near the mouth (cold sores or fever blisters), on the cornea (keratitis), or on genital tissues. Because the virus that causes the primary lesions establishes latent infections in sensory neurons and can be reactivated by appropriate stimuli, periodic recurrences of herpetic lesions are common and present one of the troublesome aspects of infections with HSV. This said, a systemic evaluation of HSV-2 in asymptomatic individuals with no reported history of genital herpes suggest a high frequency of viral shedding and ability to spread the virus even when clinical signs are not visible (Wald et al., 2000). Less frequent, but with both serious long term sequelae, and some mortality, is disease affecting vital organs, including encephalitis in apparently normal adults, and disseminated disease in infants and immunocompromised individuals.

Upon a primary infection, HSV-1 invades the neurons that innervate the infected site of infection, before being transported to the neuronal nuclei. Upon entry into the neuron the virion loses its envelope and most of the tegument proteins, leaving the capsid and some associated tegument to be transported to the nucleus. Current hypothesis suggest that the route of movement is retrograde (backward movement, from neural axon to neural cell body) along the microtubules (Diefenbach et al., 2008). It has been suggested that HSV-1 replicates briefly in neurons before establishing a latent infection in which

functional viral genomes are retained in neuronal nuclei without virus production. This latent infection may persist for the life of the individual and serves as a source of virus for recurrent diseases (Steiner, 1996). Upon reactivation, enveloped virions can exit the cell body directly, or be transported along the axon in an anterograde direction (Diefenbach et al., 2008).

1.2. HSV-1 structure

Member of the *Herpesviridae* family of DNA viruses, HSV-1 carries a large linear double stranded genome of approximately 152 kb encoding approximately 80 viral genes. The viral genome is composed of unique long (U_L) and unique short (U_S) segments, both flanked by inverted repeats (McGeoch et al., 1988). The HSV-1 genes fall into three categories depending on the kinetics of their transcription: "immediate early" (IE), responsible for preparing the cell for further viral gene expression; "early" (E), involved in the viral genome replication; and "late" (L), encoding the structural proteins (Weir, 2001).

The virion carrying the viral genome is 120 – 300 nm in diameter consisting of an electron dense core containg the DNA enclosed by an icosahedral capsid. The capsid itself is surrounded by a proteinaceous layer - the tegument - and a lipid bilayer envelope on the surface containing the glycoprotein spikes (Grunewald et al., 2003) (figure 1.1). Eleven virion proteins can be found on the surface of the envelope, including glycoprotein L, gM, gH, gB, gC, gK, gG, gJ, gD, gI, and gE. These proteins play important roles in virus attachment to target cells, cell entry, budding , cell-to-cell spread and viral immune evasion (Steven and Spear, 1997; Friedman, 2003) (Table 1.2).

1.3. Entry

HSV and other members of the herpesviruses are distinct in that they rely on three different glycoproteins (gB, gH and gL) in addition to the receptor binding gD for fusion. HSV entry occurs when extracellular virions attach to the cell's surface via gC and gB, and then bind to a gD receptor activating the membrane fusion machinery comprised of gB and the heterodimer gHgL (Campadelli-Fiume G et al., 2000; Spear, 2004) (figure 1.2). It was generally believed that HSV entry into cells occurs by direct fusion of virion envelope with the outer plasma membrane (Wittels M and Spear, 1991). Recent reports have suggested, however, that at least three diverse pathways are implicated in HSV-1 entry into different cell types that are susceptible to infection: via direct fusion with the plasma membrane, via fusion within an acidic endosome, and via fusion within a neutral endosome. Fusion at the plasma membrane is known to occur in Vero cells in a pH-independent fashion (Koyama AH and Uchida T, 1987). In most cell types virion entry is by endocytosis, but this is also variable. For example, in HeLa cells, primary human keratinocytes, and CHO-K1 cells transfected with a gD-receptor, fusion with endosomal membrane requires a low pH environment (Nicola et al., 2003; Nicola et al., 2005). In contrast virus entry in mouse melanoma B78H1 cells, expressing a human gD receptor, also involves virion endocytosis, but fusion does not require low pH (Milne et al., 2005). Furthermore, this virus internalization in B78H1 melanoma cells is completely dependent upon the expression of the gD receptor. In each of these pathways, fusion requires gD, gB, gHgL and a gD receptor (Nicola and Straus, 2004).

1.3.1. Cell surface interaction – glycoprotein C (and glycoprotein B)

The initial step in HSV-1 entry into mammalian cells is the attachment of the virus to the cell surface. gC and gB interact independently with heparan sulfate (HS)

proteoglycans to promote the initial attachment (Shieh et al., 1992; Herold et al., 1994; Laquerre et al., 1998). Yet these glycoprotein–HS interactions, although important, are not absolutely essential for viral entry, at least not in the infection of cultured cells. A virus mutant at the gB polylysine sequence responsible for the gB-HS interaction is still infectious, although virus binding is reduced; a virus lacking the gC glycoprotein is also still infectious (Herold et al., 1994; Laquerre et al., 1998). If both gB and gC are absent however, virus binding to cell surface is severely reduced (Herold et al., 1994). Infectivity is abolished completely in such virions, but this could be due to gB's critical role in membrane fusion, rather than to virion attachment at the cell surface. Recent data suggest, however, that another receptor, independent of HS, may mediate interaction of gB with the cell surface, since a soluble form of gB was shown to interact with the surface of different cell types independent of HS (Bender et al., 2005). It has recently been suggested that the paired immunoglobulin like-type 2 receptor (PILR)-alpha associates with gB and mediates infection (Satoh et al., 2008). This receptor will be discussed further in section 1.3.3.

1.3.2. Entry receptor interaction – glycoprotein D

Following gC and/or gB interaction with the cell surface, the next stage in viral entry requires the interaction of gD with one of several potential entry receptors. There are currently several known classes of receptors thought to play a role in entry. The differential use of these receptors is important, and may help to account for the entry of HSV into such a wide range of different cell types (Richart et al., 2003; Krummenacher et al., 2004; Tiwari et al., 2006; Taylor et al., 2007). The binding of gD with its receptors is found to be essential for viral entry. Upon binding, gD is thought to transmit a signal to gB and/or gH-gL which interact with the cellular membrane to mediate cell

fusion (Cocchi et al., 2004; Krummenacher et al., 2005). It has recently been suggested, that the role of these receptors regardless of which receptor is used, is to trigger a common mechanism necessary for fusion. Studies have shown that soluble gD receptors are still able to initiate viral entry in non-susceptible CHO cell lines, suggesting that the attachment of gD with the cell surface is not essential (Kwon et al., 2006; Tiwari et al., 2007). Detailed below are the three known receptors that bind gD.

Herpesvirus Entry Mediator (HVEM)

Member of the TNF receptor (TNFR) superfamily (Montgomery et al., 1996; Kwon et al., 1997), HVEM (also know as HveA) is expressed in a variety of tissues and cell types (Kwon et al., 1997; Tan et al., 1997; Harrop et al., 1998). The viral ligand for HVEM is gD (Whitbeck et al., 1997); HVEM's physiological ligands are the TNF-like ligands LIGHT and lymphotoxin-alpha (Mauri et al., 1998) and, according to more recent studies, also BTLA (Sedy et al., 2005; Gonzalez et al., 2005). Characteristically for TNFR the extracellular domain of HVEM contains common cysteine-rich domains (Locksley et al., 2001) which have been found to be necessary for the binding of both gD (Whitbeck et al., 1997; Whitbeck et al., 2001; Connolly et al., 2002) and of HVEM's physiological ligands (Sarrias et al., 2000). However, studies have shown that viral gD and the cellular ligands do not bind to the same region on HVEM (Compaan et al., 2005). In response to ligand binding, the cytoplasmic domain associates with TNF-receptor associated factors (TRAF), specifically TRAF1, 2, 3 and 5 (Marsters et al., 1997; Hsu et al., 1997), and the resulting signaling cascade is thought to contribute to the function and survival of many types of cells involved in immune responses (Harrop et al., 1998; Croft, 2003; Granger and Rickert, 2003). In HSV infection, HVEM plays a

crucial role in viral entry and cell-cell spread (Montgomery et al., 1996; Whitbeck et al., 1997; Roller and Rauch, 1998), and has been found to be specific for HSV gD binding.

Nectin

Two members of the immunoglobulin (Ig) superfamily, nectin-1 (also termed HveC) and nectin-2 (also termed HveB) have been identified as another group of receptors known to play a role in HSV entry. All HSV strains tested to date are able to bind to nectin-1 and trigger entry (Krummenacher et al., 2004), but only a few isolates (HSV-2 and certain mutant strains of HSV-1) were shown to utilize nectin-2 (Warner et al., 1998; Lopez et al., 2000; Connolly et al., 2003). Nectin-1 is a type I transmembrane protein (Geraghty et al., 1998) containing three Ig-like extracellular domains, one variable (V) and two constant (C), followed by a transmembrane domain (TM) and a cytoplasmic tail (CT). It is expressed on a variety of cells, including epithelial and neuronal cells (Geraghty et al., 1998; Krummenacher et al., 2000; Haarr et al., 2001; Hung et al., 2002; Richart et al., 2003; Matsushima et al., 2003), and functions as a cell adhesion molecule forming the adherens junctions between epethilial cells (Takahashi et al., 1999; Sakisaka and Takai, 2004).

In HSV infection, the N-terminal Ig-like variable domain is crucial for gD binding (Krummenacher et al., 2000; Lopez et al., 2000; Martinez and Spear, 2002; Struyf et al., 2002; Kwon et al., 2006). It has been suggested by several studies that HVEM and nectin-1 bind to closely located, yet different sites on gD, so that when the glycoprotein is in its bent structure forming a hairpin loop at the N-terminal it can bind to HVEM and in its extended form it binds to nectin-1 (Carfi et al., 2001; Connolly et al., 2005). Which of these receptors is more relevant in the infection of HSV *in vivo* is still to be determined. Although studies have shown that both receptors are capable of mediating

HSV entry, studies on receptor expression demonstrate that each molecule of nectin-1 is more efficient at promoting entry compared to HVEM (Krummenacher et al., 2004).

3-O-sulfated heparan sulfate

Another gD receptor identified to play a role in HSV-1 (but not HSV-2) entry is the non-protein 3-O-sulfated heparan sulfate, the product of certain 3-O- sulfotransferases (Shukla et al., 1999; Shukla and Spear, 2001; Tiwari et al., 2006). More recently, this product has also been shown to be important in polykaryocyte formation in human corneal fibroblasts used by the virus for cell-cell spread (Tiwari et al., 2007).

1.3.3. Fusion – glycoprotein B and the gH/gL complex

The least understood stage in HSV-1 entry into mammalian cells is the fusion of the virion envelope with the cellular membrane. In the absence of gB, gH or gL, HSV cannot enter target cells (Cai WZ et al., 1988; Forrester A et al., 1992; Roop et al., 1993; Chiang HY et al., 1994; Cocchi et al., 2004; Zago et al., 2004; Krummenacher et al., 2005). Since gD does not have the characteristics of a fusion protein, it is assumed that the central fusion machinery involves gB and the heterodimer gH/gL.

In many enveloped viruses the fusion process is performed by specific proteins in the viral membrane. To date, two completely unrelated structural classes have been identified, designated type I and type II fusion proteins. In the type I model (identified in orthomyxoviruses (Wilson IA et al., 1981; Bullough et al., 1994), paramyxoviruses (Yin et al., 2006), retroviruses (Fass D et al., 2006), filoviruses (Weissenhorn et al., 1998) and coronaviruses (Xu et al., 2004), the fusion proteins form homotrimers that are cleaved proteolytically into a surface (SU) subunit and a transmembrane (TM) subunit anchored to the viral membrane. These fusion proteins are extended to a rodlike

structure in response to an activating trigger. A hydrophobic α-helix fusion peptide at the N-terminus of the TM subunit is exposed and able to penetrate the target cell membrane. A further conformational change in the TM subunit brings together two heptad repeats (HR-1 and HR-2) located downstream of the fusion peptides, to form a coiled coil structure resulting a stable hairpin shaped conformation. This folding back of the fusion protein upon itself brings the viral and cellular membranes into close contact allowing the lipids in the outer membranes to mix (Colman and Lawrence, 2003; Jardetzky and Lamb, 2004; Schibli DJ and Weissenhorn W, 2004). In the type II model [applicable to flaviviruses (Rey et al., 1995; Modis et al., 2003) and alphaviruses (Lescar et al., 2001)], the fusion protein (which bears an internal fusion peptide able to penetrate the membrane of the target cell) is folded flat on the viral surface, in tight association with a second protein as a heterodimer. The activating cleavage occurs in the second protein. Receptor binding then leads to an irreversible rearrangement of the fusion protein into a trimer protruding from the viral envelope. This allows the penetration of the exposed hydrophobic hairpin loop into the target cell membrane (Colman and Lawrence, 2003; Jardetzky and Lamb, 2004; Kielian, 2006). A fold-back movement of the fusion protein then brings the viral and cellular membranes together.

The key role in HSV-1 fusion is thought to be played by the glycoproteins B and H, both conserved glycoproteins in the Herpesviridae family (Gompels U and Minson A, 1986; Forrester A et al., 1992; Pereira L, 1994). Which one of these is actually performing membrane fusion and whether one is supporting the fusogenic activity of the other or if they both function at different stages or together, remains unclear. However recent advances in determining the structures of the HSV fusion glycoproteins indicate that herpesviruses may use some kind of intermediate mechanism with components similar to both type I and type II.

In its organization, gH resembles viral fusion glycoproteins - a possible α-helix contained within the ectodomain of gH (at residues 377 to 397) has the characteristics of an internal fusion peptide, and two downstream heptad repeats (HR-1 and HR-2) could potentially interact and adopt a coiled coil conformation (Gianni et al., 2005a). Mutation or site deletion of the gH α-helix results in HSV's inability to enter cells; its replacement with heterologous fusion peptide of HIV gp41 or of VSV-G partially restores virus infectivity and gH fusion activity (Gianni et al., 2005b). Furthermore, disrupting the HR's capability in forming coiled coils, also affected the ability of gH to function in the infectivity and cell fusion of cultured cells (Gianni et al., 2005b; Gianni et al., 2005a). Although gH/gL do not form trimers, these mutagenesis data suggest a function for gH as a fusion protein in HSV viral entry.

This being so, gH's function is dependent on forming a heterodimer complex with gL, which acts as a gH chaperone for proper processing and gH trafficking to the viral envelope. HSV-1 gL is a short (224 amino acids) glycoprotein lacking a transmembrane domain (Peng et al., 1998a; Hutchinson et al., 1992; Dubin and Jiang, 1995). Upon expression in a complex with gH, gL is associated with the viral envelope; and, in the absence of gH, gL is secreted from cells (Dubin and Jiang, 1995). However, HSV-1 virions lacking gL also lack gH, and although they bind to the cell surface, they do not penetrate the cellular membrane (Hutchinson et al., 1992; Roop et al., 1993).

Like gH, gB is conserved among the Herpesviridae family (Pereira L, 1994). The recently solved crystal structure of HSV-1 gB (Heldwein et al., 2006) reveals a structure reminiscent of type I fusion proteins. On the other hand HSV gB is not cleaved proteolytically and may contain an internal fusion peptide similar to type II fusion proteins (Hannah et al., 2007). Studies involving virus-neutralizing monoclonal

antibodies directed towards specific sites on the gB ectodomain found that antibodies that affected fusion reacted with residues in the mid-region of the ectodomain, suggesting that the gB ectodomain is exposed on the surface of the virion and may be required for viral penetration and cell fusion (Highlander SL et al., 1988; Heldwein et al., 2006).

Interestingly, the HSV gB ectodomain structure is homologous to the fusion protein G of Vesicular Stomatitis Virus (VSV) – a rhabdovirus with single-stranded RNA genome (Roche et al., 2006). As protein G of VSV is known to be the viral fusion protein, this structural similarity strongly suggests that gB might be the effector of membrane fusion during HSV entry. Because both VSV-G and HSV-gB have structural features of both class I and class II fusion proteins, they have been proposed to represent a new class of viral fusion proteins (Roche et al., 2006).

Recent studies using cell-cell fusion and virus-cell fusion assays have shown that HSV-1-induced membrane fusion occurred through a hemifusion intermediate and that gD and gH/gL were sufficient to mediate hemifusion but not complete fusion (Subramanian and Geraghty, 2007). It is suggested that gB is necessary for completing the fusion process because complete fusion was ablated in the absence of gB. Regardless of which of gB or gH is the central fusion effector, these glycoproteins act in a complex with gD to promote virus fusion (Avitabile et al., 2007; Atanasiu et al., 2007). How they are activated by gD binding to cell receptors, and how this machinery functions, remains to be elucidated.

The receptor(s), if any, used by these viral fusion proteins is still unclear. It has been suggested, as mentioned above, that gB may interact with PILR-alpha. Studies on PILR-alpha transfectants, HSV-1 mutants, and primary cells expressing both gD receptors and

PILR indicated that both gB and gD receptors are required for HSV-1 infection (Satoh et al., 2008). However, to date there is no data which demonstrates the role of this receptor in fusion. This being so, several studies have tried to identify a cellular receptor for the complex gH/gL. The gH ectodomain contains a potential integrin-binding motif, thought to be responsible for the binding to cells. Indeed, modified gH/gL with a mutation in the integrin-binding-motif, abolished binding of gH to Vero cells; and CHO cells expressing the human αvβ3-integrin bound efficiently to the gH/gL complex, suggesting the gH can bind to cells via αvβ3-integrin (Parry et al., 2005). Recently, a new human protein, designated B5 was identified, which may function as an additional co-receptor for HSV entry (Perez-Romero and Fuller, 2005). This protein was found to contain hepatad repeats at the extracellular C terminus that are predicted to form an α-helix for coiled coils, similar to those in the cellular fusion proteins SNAREs, or in some viral fusion proteins. It is speculated that the complex gH/gL, which also form α-helices, may be the possible ligands for B5.

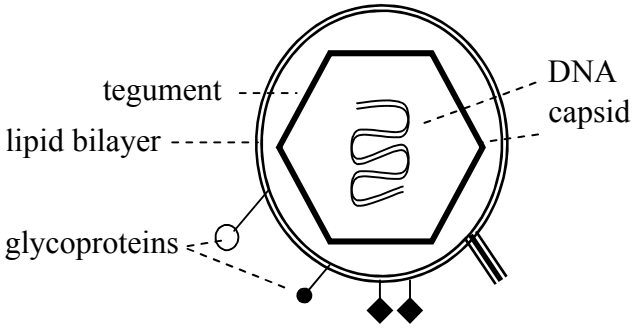


Figure 1.1: Schematic diagram of the HSV virion. The double stranded DNA is enclosed within an electron dense core enclosed within an icosahedral capsid. The capsid itself is surrounded by a proteinaceous layer - the tegument - and a lipid bilayer envelope on the surface containing the glycoprotein spikes.

Glycoprotein	Gene	Function
L	U_L1	Viral entry
M	U_L10	Virion assembly
H	U_L22	Viral entry
B	U_L27	Viral entry
C	U_L44	Virus-cell attachment; inhibition of complement
K	U_L53	Intracellular trafficking; viral budding
G	U_S4	Uknown function
J	U_S5	Protection from CTL apoptosis
D	U_S6	Viral entry
I	U_S7	Associates with gE; cell-cell spread; inhibition of antibody attack
E	U_S8	Associates with gI; cell-cell spread; inhibition of antibody attack

Table 1.2: HSV-1 glycoproteins and their function

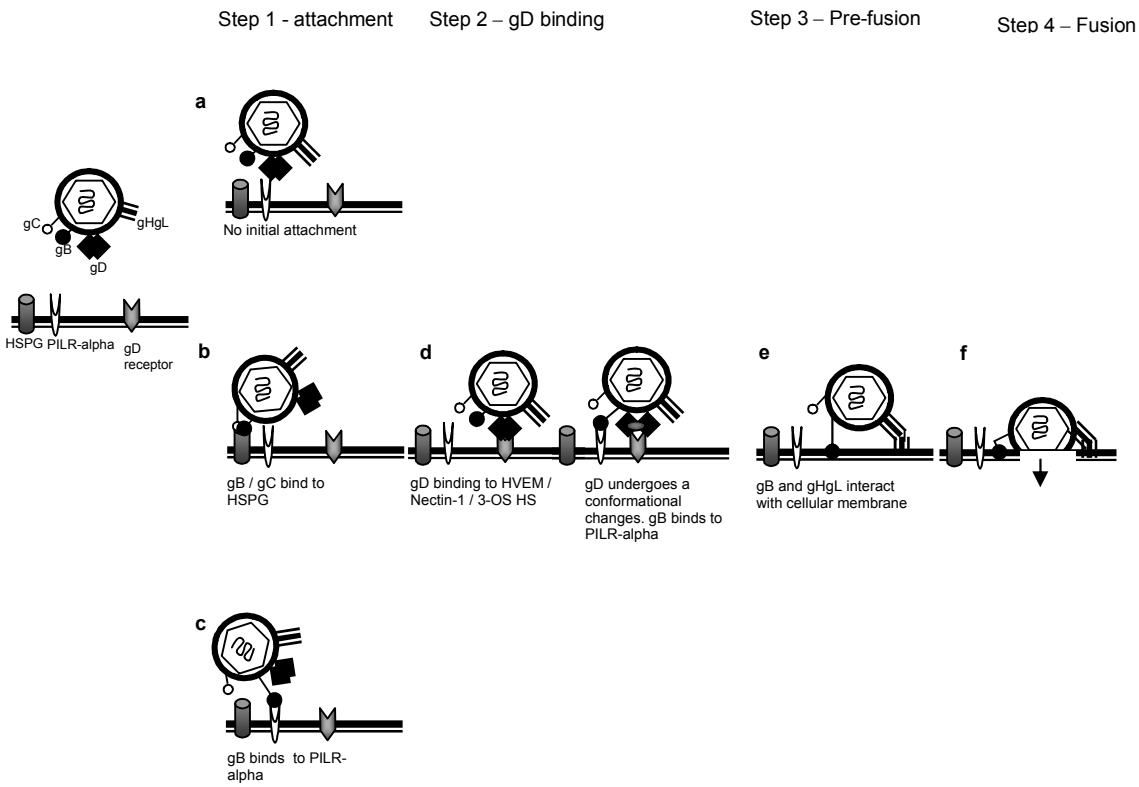


Figure 1.2: Model for direct HSV-1 entry by fusion with the cellular membrane. Initially, although not mandatory (a), gB (together with gC) binds with heparan sulfate proteoglycans (HSPG) (b) or with an unknown gB receptor, independent of HS (c), allowing virus-cell attachment. This is followed by gD binding to a cell surface receptor: HVEM, nectin-1 or 3-O sulfated heparan sulfate (d). Then gD undergoes conformational changes and transmits a signal gB and/or gH-gL. gB possibly interacts with an unknown cell surface component. gB and gH-gL form the fusion machinery that interact with the cellular membrane (e). gB and gH-gL mediate cell fusion and entry of the viral capsid (f). How gB, gH and gL work together to achieve fusion is unclear and steps 3 and 4 are drawn hypothetically.

1.4. Immune response to HSV-1

The outcome of an HSV infection is partly determined by the host immune response. It is well established that immunocompromised individuals are more susceptible to viral infection. The response to HSV occurs in two phases, the innate and adaptive immune response. Traditionally, the innate immune system was considered as simply the non-specific first line of defence. It is in the past decade or so, that more studies have suggested that despite its non-specificity, the innate immune response can distinguish between self and the infectious non-self and can be mobilised upon infection. The adaptive immune response on the other hand, acts as a second line of defence and affords a more pathogen specific protection as well as protection against re-exposure to the same pathogen.

1.4.1. Innate immune response

Innate immune response against HSV-1 includes the generation and secretion of a variety of cytokines and chemokines which are responsible for the action of the innate immune cells. These cells act to limit the initial infection of the tissue, abrogate further propagation of the virus, and begin antigen presentation to initiate the activation of specific T and B cell response. The mechanisms used by the host in achieving these results are summerized below.

Skin and mucosal membrane

In addition to its function as a physical barrier to penetration, the skin as the body's first line of defence against infection, includes at least two major functional immune components: the innate-inflammatory immunity, which involves the recognition of microbial compounds and the subsequent expression of pro-inflammatory cytokines and

interferons; and the “early warning” system which attracts the adaptive immune response (Meyer T et al., 2007).

In natural human infection, HSV’s primary site of replication is within mucosal or epithelial cells. In the skin, epidermal keratinocytes play a vital role in the defence against the virus with the dermis as the site of the main inflammatory infiltrate (Cunningham AL and Noble JR, 1989). Studies have shown that infected keratinocytes express MHC class II antigens within 2 days of infection (Cunningham AL et al., 1985) with production of various cytokines and chemokines, including beta chemokines, IL-12, IL-10, IL-1alpha, IL-1beta and IL-6, early in the infection (Mikloska Z et al., 1998). It has been suggested that upon an infection the non-immune cells are primed to respond to low levels of viral infection by the induction of a small subset of interferon stimulating genes (ISG) which act to block viral replication without causing cellular damage. If, however, a threshold of viral entry is exceeded, interferon and proinflammatory cytokines are secreted warning surrounding cells to induce an antiviral response and to attract immune cells to the site of infection (Collins et al., 2004).

NK cells

Natural killer (NK) cells are critical components of the innate immune system and have key roles in early immune responses to various pathogens (Yokoyama et al., 2004). Bone-marrow derived, they are capable of lysing cells without prior sensitization and release cytokines important in the regulation of the adaptive immune response. Upon a viral infection they undergo both nonspecific and viral specific proliferative phases, the latter related to NK cell-activation receptors for infected cells (Dokun et al., 2001; Yokoyama et al., 2004; Yokoyama et al., 2004). Severe infections with HSV have been observed in individuals lacking NK cells (Biron CA et al., 1989; Orange, 2002; Dalloul

et al., 2004) and several studies both in vitro and in vivo have demonstrated a NK cell role in innate response to HSV-1 (Fitzgerald et al., 1985; Rager-Zisman et al., 1987; Baraz L et al., 1999; Tanigawa et al., 2000). Recent reports, however, have suggested otherwise, demonstrating that immediate innate immune resistance to HSV infection, at least in the mouse model, is independent of NK cells (Vollstedt et al., 2004; Halford et al., 2005).

Macrophages

Macrophages play an important role in the host defence mechanism. As phagocytic cells, macrophages act both in the initial killing defence and as activated antigen presenting cells. The role played by macrophages in the immune response to viruses is well established (Mogensen, 1979). Murine studies have also shown the presence of non-infected macrophages at the site of infection. These cells were found to be responsible for the secretion of TNF-alpha and type I IFN, necessary for the anti-viral response (Fields et al., 2006) and in the case of TNF-alpha, as a key mediator in the recruitment of cells to the site of infection, specifically via its effect on the local blood vessel endothelium and endothelial leukocyte interaction (Bradley JR, 2008).

HSV-1 has also been shown in several studies to infect macrophages both *in vitro* and *in vivo*, but HSV does not actively replicate in either murine or human macrophages (Linnavuori K and Hovi T, 1983; Brucher J et al., 1984; Sarmiento, 1988). Nonetheless, infection of macrophages with HSV results in the partial activation of the cells as measured by upregulation of surface markers, low nitric oxide production and cytokine/chemokine secretion (Ellermann-Eriksen, 1993; Croen KD, 1993; Heise and Virgin, 1995; Paludan and Mogensen, 2001; Malmgaard et al., 2000; Paludan SR et al.,

2001; Malmgaard et al., 2004), yet decreased ability to stimulate T cells (Hoves et al., 2001).

Dendritic Cells

Dendritic cells (DC) are the most potent antigen presenting cells (APC) with a remarkable ability to stimulate naïve T lymphocytes. Residing in most peripheral tissues throughout the body, in the absence of an inflammatory and immune response, immature DC function to sample and capture foreign antigens in the periphery. Upon a signal from a pathogen – whether by direct recognition of pathogens (through specific pattern-recognition receptors) or indirect sensing of infection (through inflammatory cytokines, internal cellular compounds, or ongoing specific immune responses), DC mature, expressing higher levels of cell surface MHC class I and II, which transforms them into efficient T cell activators, while at the same time losing their ability to pick up any more antigen. As non-proliferating cells, after a certain time course, these cells undergo apoptosis to be replaced by different cells (Banchereau and Steinman, 1998; Banchereau et al., 2000). A unique feature of DC is their ability to take up and present endosomal peptide fragments and deliver to the MHC class I, instead of the conventional route for exogenous antigens, which is to the MHC class II. This results in CD8 T cell priming. The process is known as cross-presentation (Guermonprez et al., 2005), and has several advantages for the host in viral infections, as DC can take up and and present viral peptides from infected cells and then induces a cytotoxic T cell response. As these cells (DC) are the primary focus of the work described in this thesis, their biology will be discussed in more detail in section 1.6.

It is now well established that HSV infects human DC (Salio et al., 1999; Kruse et al., 2000; Pollara et al., 2003). Infection of immature DC by HSV-1 leads to a series of both

morphological and functional changes including partial maturation (as judged by surface phenotype), impairment of antigen presenting function and eventual apoptosis. However, the initial effect of HSV on DC, at least in monocyte derived DC (MDDC) *in vitro*, is the release of type I IFN (Pollara et al., 2004a). Studies have shown that type I IFN released by HSV infected DC, in addition to its anti-viral activity, also acts as a danger signal to bystander uninfected DC, inducing maturation, release of IL-12 and migration of DC cross-presenting antigen, which in turn induces paracrine maturation of further uninfected DC (Luft et al., 2002; Pollara et al., 2004a). This would promote a more effective T_H1 immune response, and provide the link between the innate and adaptive phases.

Several pieces of evidence point to a role for DC in HSV-1 infection *in vivo*. In cutaneous HSV-1 infection, a large number of both myeloid DC and plasmacytoid DC precursors were shown to appear de novo in the circulation (Yoneyama et al., 2004a). Studies on mice of the C57 background (C57BL/6 and C57BL/10), shown to be the most resistant and best able to control HSV-1 infections (Simmons and Tscharke, 1992), demonstrate the key role of these cells in the resistance to HSV-1 infections. Ablation of CD11c DC in these resistant strains of mice lead to the enhanced susceptibility to HSV-1 infection, including an increase in viral spread to the nervous system and an impaired activation of NK cells and both $CD4^+$ and $CD8^+$ T cells (Kassim et al., 2006). While pDC and to some extent mDC were shown to be responsible for the type I interferon production in response to HSV-1 infection (Lund et al., 2003; Krug et al., 2004; Hochrein et al., 2004).

It is well established that DC express receptors known to associate with HSV-1 glycoproteins, specifically HVEM and nectin-1 (Salio et al., 1999) and the different toll-

like receptors (TLR). Recent data demonstrate a role for DC-SIGN in the interaction with gB and gC, although this receptor was shown not to be an entry receptor (de Jong MA et al., 2008). And yet, the route by which HSV-1 activates DC has not been fully characterized.

Cytokines and chemokines

Cytokines are important factors in the immune response and the protection against viral infections (Table 1.3). Yet, cytokines are also involved in the pathogenesis and development of symptoms of infection. Probably the most powerful anti-viral cytokines are the **type I interferons (IFN)** comprised in humans of 14 IFN-α isoforms, a single IFN-β and several other members such IFN-ω, -ε, -κ (Honda et al., 2006; Stetson and Medzhitov, 2006; Garcia-Sastre and Biron, 2006). In addition to their classical antiviral properties, type I IFN also contribute to the antiviral immune response by stimulating the cytotoxic activity of NK cells, upregulation of MHC class I, maturation of bystander uninfected DC, and promotion of various T-cell functions, including expansion of the memory cells (Garcia-Sastre and Biron, 2006). Virtually all cells are able to produce type I IFN in response to viral infection, but plasmacytoid DC (discussed further in section 1.6.2) have been identified as the cells responsible for the majority of IFN production (Siegal et al., 1999; Asselin-Paturel et al., 2001; Colonna et al., 2002). In HSV-1 infection, type I IFN produced early in the infection by different cell types is known to block viral replication and to be important for the resistance against the virus (Mittnacht S et al., 1988; Leib et al., 1999; Noisakran et al., 2000). Defects in the IFN pathway in children with autosomal recessive deficiency of the intracellular protein UNC-93B have been shown to be linked with susceptibility to HSV encephalitis (Casrouge et al., 2006).

Type II IFN is a single cytokine, IFN-γ. Secreted primarly by NK cells, $CD4^+$ and $CD8^+$ T cells, IFN-γ has a wide range of immunomodulatory effects on both the innate and the adaptive immune response (Farrar and Schreiber, 1993). Although known to have direct anti-viral activity, IFN-γ is known more for its immunoregulatory functions (Boehm et al., 1997; Nansen et al., 1999; Henrichsen et al., 2005). Studies on null-mutant mice (Bouley et al., 1995; Cantin et al., 1999) and human fibroblasts (Peng et al., 2008) have confirmed the importance of IFN-γ in host immune response to viral infections, including HSV-1.

More recently, a **type III IFN** (IFN-λ) was discovered which has been shown to have anti-viral activity, via a mechanism similar to, but independent of, type I IFN (Kotenko et al., 2003; Sheppard et al., 2003). Studies on IFN-λ have indicated that not all viruses are susceptible (Ank et al., 2006) and not all cell types are responsive to this cytokine (Sheppard et al., 2003; Kotenko et al., 2003). However, the antiviral activity of IFN-λ against HSV-2 has been shown to be comparable to that of IFN-α in a systemic model (Ank et al., 2006; Ank et al., 2008).

The **tumour necrosis factor (TNF)** superfamily of cytokines plays an essential role in the early innate and subsequent adaptive immune responses against infections. The family includes several pro-inflammatory cytokines secreted by various different cell types. Pre-treatment of mice with TNF-α has been reported to provide significant protection to lethal HSV-1 challenge in a pathway independent of interferon (Rossol-Voth et al., 1991). Furthermore, TNF-α deficient C57BL/6 mice show an increase in lethal encephalitis and viral replication in the brain (Sergerie et al., 2007). In humans, in-vitro studies on HSV-1 infected corneal epithelial cells demonstrate a rapid

upregulation of pro-inflammatory cytokines, including TNF-α (Li H et al., 2006), suggesting a possible role for this cytokine in-vivo in resistance to HSV.

Other pro-inflammatory cytokines known to play a part in viral immunity include **IL-1β**, **IL-6**, and **IL-12**. In animal models, these cytokines have been shown to be secreted following HSV infection predominantly by lymphocytes, monocytes and macrophages. Studies have shown that upon HSV infection IL-1 and IL-6 are induced peaking at day 10 post-infection (Shimeld et al., 1997). Studies on IL-1β knock-out (Sergerie et al., 2007) and IL-6 knock-out (LeBlanc et al., 1999) mice demonstrate the importance of these cytokines in protection against HSV infection in the mouse model.

IL-12 is a heterodimer produced mainly by activated inflammatory cells in response to microbial infections to favour a Th1 response (Manetti R et al., 1993). Early *in vitro* (Macatonia et al., 1995) and *in vivo* (Sousa et al., 1997) studies demonstrated rapid IL-12 production by DC independent of IFN-γ or T cells. This being said, T cells, by direct contact and/or by IFN-γ and IL-4 production, enhance the IL-12 production by DC (D'Andrea A et al., 1995; Marshall et al., 1997; Cella M et al., 1996; Kalinski et al., 2000). Produced by APC mainly via the TLRs (Medzhitov and Janeway, 1997), in humans, DC, but not pDC are the main producers of IL-12 in response to microbial molecules (Kadowaki et al., 2001).

Chemokines, like cytokines are involved in the early host response to viral infection, with the inflammatory chemokines responsible for the attraction of immune cells. Several studies have looked at the chemokine response following HSV infection, demonstrating chemokine and chemokine receptor expression at both primary and latent infection sites, resulting in the recruitment of cells to and from the sites of infection (Cook WJ et al., 2004; Araki-Sasaki et al., 2006).

Cytokine	Producing Cells	Function(s)
IL-1α/β	monocytes; macrophages; DC	pro-inflammatory; stimulation of thymocytes proliferation; B cell maturation and proliferation
IL-2	T_H1 cells	Immunoregulatory; proliferation and activation of B, T and NK cells
IL-4	T_H2 cells	differentiation of naïve $CD4^+$ to T_H2 cells; proliferation of activated B and T cells
IL-6	T cells; monocytes; fibroblasts; keratinocytes	pro-inflammatory / anti-inflammatory; B cell differentiation and antibody production; inhibition of TNF-α and IL-1 and activation of IL-10
IL-10	monocytes; macrophages; DC; T and B cells; keratinocytes	anti-inflammatory; inhibiting synthesis of pro-inflammatory cytokines; ability to suppress antigen presentation by APC; enhances B cell proliferation and antibody production
IL-12	monocytes; macrophages; DC; B cells	pro-inflammatory; differentiation of naïve T cells to T_H1 cells; stimulates production of IFNγ and TNFα from T cells and NK cells
IL-13	T_H2 cells	anti-inflammatory effects on monocytes and macrophages; inhibits expression of pro-inflammatory cytokines; important mediator in allergic inflammation
IL-17A	T_H17 cells	T cell dependent inflammatory response; production of pro-inflammatory cytokines
IL-23	DC	stimulates naïve $CD4^+$ T cells to differentiate to T_H17 cells
IFN-α/β	almost all cells	anti-viral activity; maturation of bystander DC; up-regulation of MHC; stimulation of T and NK cells
IFN-γ	T_H1 cells; NK cells	Immunoregulatory functions; increases antigen presentation on macrophages; suppresses T_H2 activity
IFN-λ		anti-viral activity; upregulation of MHC antigen expression
TNF-α	monocytes; macrophages; DC; B and T cells	pro-inflammatory; regulation of immune cells

Table 1.3: Selected cytokines and their functions.

1.4.2. Adaptive immune response

As mentioned, HSV has evolved several mechanisms to evade and inhibit the functions of both T and B cells. And yet, the appearance of HSV-specific antibodies and HSV-specific T cells with cytotoxic function against infected cells suggest a vital function for the adaptive immune response in viral clearance.

Antibody response

Neutralizing antibodies following HSV infection have been documented for many years. In animal models antibody elicited by natural infection or immunization has been shown to modulate viral pathogenesis (McDermott MR et al., 1989; Bourne et al., 2003). However, evidence for the protective effect of antibodies in humans is controversial: studies of neonatal infections have shown maternal antibody protection against viral transmission at birth in some cases, but not others, with antibodies specific to HSV-2 giving a better protection against neonatal transmission of HSV-2 than HSV-1 (Brown ZA et al., 1991; Brown et al., 2007). Immunization with a HSV vaccine comprising recombinant HSV-2 gB and gD glycoproteins elicited high titers of neutralizing antibodies, but a modest level of protection, possibly due to a low antibody-dependent cellular cytotoxicity (ADCC) observed (Corey et al., 1999; Stanberry et al., 2002).

Antibody response has been detected against a wide array of viral proteins including envelope glycoproteins, tegument and capsid proteins (Ashley R et al., 1985; Ashley RL et al., 1994). Animal experiments suggest that viral glycoprotein gD induces neutralizing antibodies and several recombinant glycoprotein vaccines have been developed based on these findings (Cohen GH et al., 1972; Langenberg et al., 1995; Corey et al., 1999; Bernstein and Stanberry, 1999; Bourne et al., 2003). Furthermore,

mice immunized passively with monoclonal antibodies to gB, gC, gD and gH or immunized actively with either recombinant or synthetic viral glycoproteins were protected from lethal virus challenge (Balachandran N et al., 1982; Chan et al., 1985; Eisenberg RJ et al., 1985). HSV-1 specific neutralizing antibodies were also obtained in response to the gH-gL heterodimer, although their protective efficiency against viral challenge varied considerably in published studies (Browne H et al., 1993; Peng et al., 1998b). However despite this, the development of an effective HSV vaccine based on antibody production remains elusive.

CD4+ T cell response

CD4 T cells play an important role in the immune response. They express surface co-stimulatory molecuelsm and produce an array of cytokines, which amplify the responses of other cells involved in both the innate and adaptive immune response. Three distinct subsets of activated cells are characterised by their abilities to produce different cytokines and by the function they perform. The differentiation (or polarisation) from the naïve CD4 cell to a specific subset is itself regulated by specific cytokines (figure 1.3) (Jelley-Gibbs et al., 2008). The best characterised are the pro-inflammatory, IFN-γ producing T_H1 and the anti-inflammatory, T_H2 subsets. Although, several studies have suggested a defence role for the T_H2 subsets as well, specifically in parasitic helminths infections (Anthony et al., 2007). More recently a separate CD4 T cell subset has been identified, the IL-17 producing T_H17, which is thought to play a part in the generation of auto-immunity as well as in the protection against pathogens such as *Bordetella pertussis*, *Listeria monocytogenesis*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Candida albicans*, to name just a few (Khader et al., 2007; Curtis and Way, 2008).

Another CD4 T cell subset, independent of the T_H1, T_H2 and T_H17, are the $CD4^+CD25^+$Foxp3 regulatory T cells (Treg), whose function is to maintain immune system tolerance to self antigens. This is achieved by direct elimination of the cytotoxic cells, inhibition of proliferation and/or cytokine production in pathogenic T cells, as well as production of immunoregulatory cytokines such as TGFb and IL-10 (Askenasy et al., 2008).

In HSV infection CD4 T lymphocytes are important mediators of immunity. Studies have shown the importance of $CD4^+$ cells in the protection of mice from HSV challenge (Manickan and Rouse, 1995; Milligan et al., 1998; Ghiasi et al., 2000; Harandi et al., 2001). In humans, severe herpetic infections are seen in immunocompromised individuals with impaired T cell immunity (i.e. AIDS and transplant patients) (Chrétien F et al., 1996). $CD4^+$ cells are stimulated following an HSV infection. They have been shown to present early in an infected epidermis secreting a variety of cytokines, the most important of which being IFN-γ (Mikloska Z et al., 1998). *In vitro*, IFN-γ partially reverses the down-regulation of MHC class I, permiting lysis of HSV-infected cells by CD8 cytotoxic T lymphocytes (Tigges et al., 1996), and induces MHC class II in epidermal cells rendering them more detectable by $CD4^+$ cells (Mikloska Z et al., 1996). *In vivo*, presence of high levels of IFN-γ was found to be important in the control of HSV infection, since mice lacking the ability to produce IFN-γ were more susceptible to infection (Yu et al., 1996).

CD8+ T cell Response

Although infilitration of $CD8^+$ cells to the site of infection occurs after the infiltration of $CD4^+$ cells (Cunningham AL et al., 1985), studies of immunodeficient individuals suggest the the severity of HSV disease could be inversely correlated with the number

of HSV-specific $CD8^+$ T cells (Posavad et al., 1997). Infection with HSV involves the activation of $CD8^+$ T cells within the draining lymph nodes followed by their migration into the sites of infection where they act to clear the virus (Coles et al., 2002; van Lint et al., 2004). Studies have shown that these cells play a critical role in the primary antiviral immune response (Simmons and Tscharke, 1992; Dobbs et al., 2005) and in the latent viral reactivation in sensory neurons (Liu et al., 2000; Khanna et al., 2003).

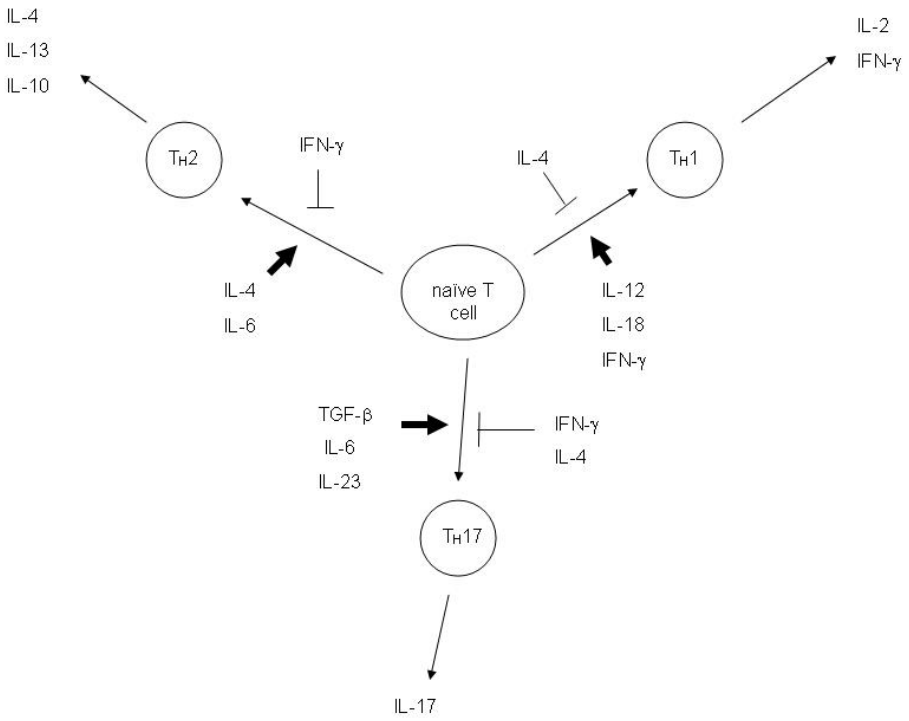


Figure 1.3: Polarisation of helper CD4 T cells.

Helper CD4 T cell subsets, showing the cytokines responsible for their induction, and the effector cytokines that they produced. Cytokines that induce polarisation are shown with (↑), cytokines that inhibit polarisation are shown with (⊤)

1.5. Evasion of the immune response

The survival of a virus depends on the balance between the virus and the host. One strategy evolved by the herpes viruses is the development of a latent infection, hence "hiding" the virus from the immune response. But before the virus infects cells such as neurons to establish latency, it has to evade the early host response. Like many other viruses, HSV has evolved a series of mechanisms for the evasion and control of the host's early immune system. Indeed the evolution of multiple viral immunomodulatory proteins suggests the immune system plays a major role in the HSV life cycle. The viral proteins with a possible role in immune evasion by HSV are discussed below:

1.5.1. Inhibition of cytotoxic T cell activity

Cytotoxic T cells (CTLs) function to kill other "target" cells, this by inducting apoptosis of infected cells, and/or by the production of anti-viral cytokines. Avoiding recognition by CTLs is an attractive mechanism for immune evasion by pathogens. HSV has evolved several mechanisms to withstand the action of CTLs, including the Us5 viral gene product, glycoprotein J which functions to suppress CTL induced apoptosis in a cell-type specific manner (Koyama and Miwa, 1997; Jerome et al., 1998; Jerome et al., 2001), hence allowing the virus to complete an efficient replication cycle. Alternatively, HSV-1 infected CTL are silenced via fratricide-induced apoptosis (Raftery et al., 1999). This is a mechanism in which HSV infection of activated CTL results in these cells being more susceptible to "self-killing" by other HSV-specific CTL because MHC class I antigen presentation is not blocked in these cells following HSV infection. HSV induction of cell apoptosis has also been demonstrated in infected activated T cells (Ito M et al., 1997; Ito et al., 1997; Pongpanich A et al., 2004; Han et al., 2007), infected B

cells (Han et al., 2007), infected DCs (Pollara et al., 2003; Muller DB et al., 2004; Bosnjak et al., 2005) and infected macrophages (Fleck M et al., 1999).

1.5.2. Inhibition of antigen presentation

Decreased antigen presentation can prevent both $CD8^+$ and $CD4^+$ T cell recognition of infected cells. One mechanism developed by the virus involves the decrease in maturation of DCs, the most potent APCs (Pollara et al., 2004a; Salio et al., 1999) (discussed in detail in section 1.6). Other mechanisms involve a more specific decrease in expression and/or antigen presentation via both MHC class I and class II. These are schematically summarised in figure 1.4.

The US12 gene (immediate early) of HSV-1 encodes infected cell protein (ICP) 47. This protein was found to interfere directly with MHC class I antigen presentation by binding to the cytosolic side of the transporter associated with antigen processing (TAP) molecules on the endoplasmic reticulum (ER), and hence, interfering with the translocation and loading of the peptides and antigen presentation to $CD8^+$ T cells (Hill et al., 1995; Fruh et al., 1995; Ahn K et al., 1996; Tomazin R et al., 1996). Recombinant ICP47 was found to protect HSV-infected human fibroblasts from CTL (Jugovic et al., 1998). The role played by ICP47 *in vivo* is unclear, as this protein inhibits TAP little, if at all, in animal models (mouse, rat, guinea pig, or rabbit) unless applied at very high concentrations (50- to 100-fold higher than those required to inhibit human TAP) (Fruh et al., 1995; Ahn K et al., 1996; Tomazin et al., 1998; Jugovic et al., 1998). Nonetheless, HSV-1 lacking ICP47 showed reduced neurovirulence in mice with functional $CD8^+$ T cells, and characteristic neurovirulence in $CD8^+$ T cell-deficient mice (Goldsmith et al., 1998). In order to address the limitations of *in vivo* studies, mice were infected with recombinant HSV expressing MCMV m152 or HCMV U11, two

herpesvirus proteins known to inhibit antigen presentation by murine MHC class I (Machold et al., 1997; Wagner et al., 2002). These rHSVs demonstrated increase in neurovirulence compared to wild-type virus, but no differences were seen in infection of MHC class I-deficient mice, or in mice in which $CD8^+$ T cells were depleted (Orr Mark T et al., 2005). This suggests that the increase neurovirulence in murine HSV infection may be due to inhibition of target cell recognition by antigen-specific CD8 cell.

MHC class II is responsible for presentation of viral proteins that are internalized and degraded by antigen-presenting cells, hence initiating the $CD4^+$ T cell response (Germain and Margulies, 1993). Several studies have shown that HSV-1 glycoprotein B contains a MHC class II binding site sequence which inhibits peptide loading and prevents presentation of viral peptides (Sievers et al., 2002; Neumann et al., 2003).

The mechanism of blocking peptide loading on to the MHC is not uncommon in human viruses (Vossen et al., 2002; Loch and Tampe, 2005). HCMV, another member of the *Herpesviridae* family, has been shown to use the US6 protein in inhibiting peptide transport via TAP (Kyritsis et al., 2001; Dugan and Hewitt, 2008). Other methods of MHC class I disruption include preventing initial proteosomal degradation (EBV, EBNA-1; HCMV, pp65); inhibiting the assembly of the MHC class I in the ER by degradation (HCMV, US2 and US11; HIV, Vpu; HHV-7, U21); inhibiting the transport of the MHC class I to the cell surface by retaining the MHC molecule in the ER (HCMV, US3, Us10; Adenovirus E19) and the Golgi (VZV, ORF66); and downregulating MHC class I molecules on the cell surface (KSHV, K3 and K5; HIV, Nef). Evasion mechanisms involving the MHC class II include, inhibition of MHC class II expression and processing.

1.5.3. Inhibition of complement activation

The complement system plays an important role in removing infected cells by activating the complement cascade. Viruses have evolved several interference mechanisms to overcome this effect, including incorporating cellular componenets that protect the virus from the complement (Vaccinia); secreting complement-control proteins which can block the complement activity (Vaccinia and Variola).

With respect to HSV-1, glycoprotein C is a receptor for the C3b fragment of the third component of complement (Friedman et al., 1984), reducing the activation of the alternative complement pathway by interfering with the C3b-C5 and the C3b-properdin interaction (Fries et al., 1986). In vitro, gC protects HSV infected cells from complement-mediated lysis (Harris SL et al., 1990); in vivo studies have also shown a role in protecting the virus against complement attack (Lubinski et al., 1998; Lubinski et al., 1999), consistent with a role for gC in evasion of the immune response and viral survival.

1.5.4. Inhibition of antibody attack

Binding of antibodies to epitopes on the surface of infected cells results in eventual lysis. In order to evade this pathway, viruses have evolved proteins (v-FcR) that bind to the Fc region of the host IgG, hence preventing free IgG to act on free virus or on viral infected cells. With respect to HSV-1, glycoprotein E and I form a hetero-oligomer complex which functions as a receptor (FcR) for the binding of the immunoglobulin G (IgG) by the Fc domain (Johnson et al., 1988; Bell et al., 1990). The anti-HSV IgG binds to the Fc receptor by its Fc end and to its target by its Fab end in a process called antibody bipolar bridging (Frank and Friedman, 1989; Van Vliet KE et al., 1992; Sprague Elizabeth R et al., 2006). Expression of gE alone is sufficient for low affinity

binding of IgG, but it is the gE-gI complex which allows for high affinity binding (Johnson et al., 1988; Dubin et al., 1990). This process has been shown to protect the virus and infected cells from the IgG-mediated immune response and complement-dependent neutralization and antibody-dependent cell-mediated cytotoxicity (Frank and Friedman, 1989; Dubin et al., 1991). Using an HSV-1 mutant that lacked the viral FcR, this viral-host complex was shown to be important for viral immune evasion in vivo as well, since mutated virus was more susceptible to anti-viral IgG than wild-type virus (Nagashunmugam et al., 1998; Lubinski et al., 2002).

Other viruses, such as HCMV and MCMV have evolved similar mechanism by inducing the expression of surface glycoproteins which can bind to the Fc region of the host immunoglobulin (HCMV, TRL11/IRL11) (Lilley at al., 2001)

1.5.5. Destabilization of cellular mRNA

Many viruses induce upon infection inhibition of cellular functions in order to establish a cellular environment favourable for viral replication. With respect to HSV, infection results in the shutoff of host cellular protein synthesis soon after viral entry (Sydiskis and Roizman, 1966). Early shutoff is associated with several viral genes including the viral gene UL41, encoding the virion host shutoff (vhs) protein. This mechanism involves the degradation of cellular mRNA, resulting in the reduction of host protein synthesis by the destabilized cellular mRNA, and thus promoting the opportunity for enhanced translation of viral mRNA, and the resultant viral protein synthesis (Kwong AD et al., 1988). It has been suggested that vhs plays a part in the viral immune evasion mechanism by suppression of pro-inflammatory chemokines and cytokines (Suzutani et al., 2000), reduction in the levels of MHC class II (Trgovcich et al., 2002) and changes in MDDC function (Samady et al., 2003).

ICP27, a multifunctional regulatory IE protein, has also been reported to be a mediator of HSV-induced immune evasion. It has been implicated in shutdown of host protein synthesis, host mRNA splicing inhibition and repression of host-gene transcription and mRNA stability (Hardwicke and Sandri-Goldin, 1994; Mogensen et al., 2004). More recent studies suggest a role in the decrease of STAT-1 phosphorylation, hence partially blocking STAT-1 translocation to the nucleus and production of a late IFN-α (Johnson KE et al., 2008). In human macrophages, ICP27-deleted mutant virus clearly induced higher expression of IFN and chemokine genes than the wild-type virus (Melchjorsen et al., 2006). Other viral genes associated with destabilization of cellular mRNA include ICP4 acting to decrease host cell mRNA stability, thus contributing to shutoff of host protein synthesis, and to the inhibition of cytokine production (Mogensen et al., 2004; Melchjorsen et al., 2006); ICP0 which has been shown to inhibit nuclear accumulation of IFN regulatory factor (IRF)-3, as well as to interfere with IFN signaling (Mossman et al., 2000; Eidson et al., 2002; Harle et al., 2002; Melroe et al., 2004); and ICP34.5 and Us11, both expressed at late stages of infection acting on the cellular RNA-activated protein kinase R (PKR), to prevent the shutoff of protein synthesis (He et al., 1997; Poppers et al., 2000).

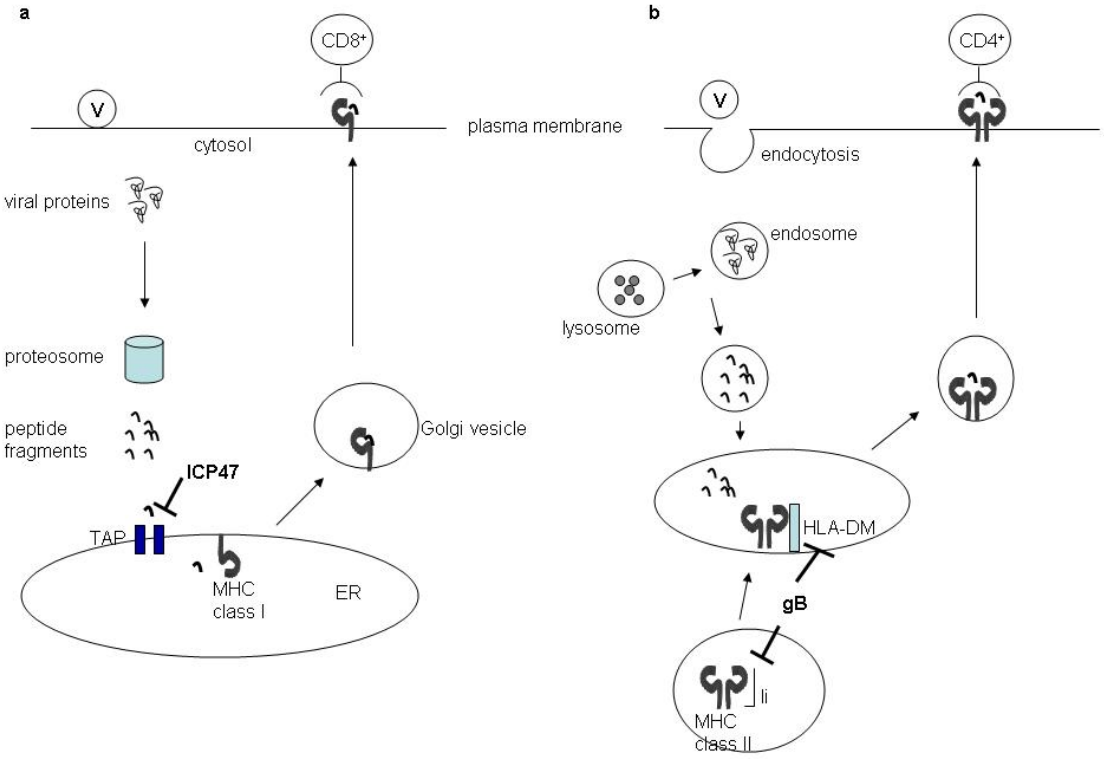


Figure 1.4: Schematic diagram of the MHC pathways, showing potential sites of HSV-1 interference. a) Viral proteins in the cytosol are degraded by the proteosomes to small peptide fragments to be transferred to the ER via TAP. In the ER, these fragments are loaded on to the MHC class I to be presented on the plasma membrane to $CD8^+$ T cells. The ICP47 protein of HSV-1 may bind to the cytosolic side of TAP, hence interfering with the peptide fragment translocation to the ER and subsequent loading to MHC class I. b) Virus entering the cell via endocytosis go through a process of lysosomal degradation and subsequent loading on to the MHC class II to be presented on the plasma membrane to $CD4^+$ T cells. It has been suggested that HSV-1 gB contains a binding motif to MHC class II invariant chain (li) and HLA-DM, thus interfering with the correct function of the MHC and subsequent viral peptide loading.

1.6. Dendritic cells

As mentioned above, DCs are the most potent APCs, active in the innate immune response and in the initiation of the adaptive immune response. It is well established today that DCs are activated via receptors, termed pattern recognition receptors (PRR) that recognize conserved pathogen associated molecular patterns (PAMP) - components commonly found on the pathogen that are not normally found in the host. In DCs, the PRR are chiefly members of the Toll-like receptor (TLR) family, although other receptors, including complement receptors (CR3; CD11b/CD18 and CR4; CD11c/CD18) (Thieblemont N et al., 1993; Bajtay et al., 2006), scavenger receptors (Mukhopadhyay S and Gordon S, 2004) and C-type lectins (Cambi and Figdor, 2003; Ebner et al., 2004) are also known to trigger activation of DC. There are currently 10 known TLR in humans, and the number of known ligands for each receptor is increasing (Table 1.3). Studies of DC subsets isolated from humans and mice have suggested a difference in TLR expression on different subsets (Iwasaki and Medzhitov, 2004; Flacher et al., 2006) (discussed below). Activation of the TLR leads to the induction of an inflammatory response via several distinct signaling pathways of which most (except for TLR3) are dependent on the adapter moclecule MyD88. The existence of individual pathways for each TLR may explain the distinct responses elicited by the different TLR agonists (Takeda et al., 2003).

1.6.1. Toll-Like Receptors

Over the years, TLR recognition of viral products has been studied, and six different TLR have been associated with virus recognition (Morrison, 2004). Originally it was expected that TLR3 which recognizes double-stranded RNA, would be the principal mediator of viral recognition (Alexopoulou et al., 2001). Studies have shown, however,

that TLR7 and TLR8, which recognize single-stranded RNA, are involved in the response to influenza virus and HIV (Heil et al., 2004; Diebold et al., 2004; Lund et al., 2004) and more recently TLR7 was suggested to play a role also in MCMV (Zucchini et al., 2008); TLR4, which recognizes gram-negative bacterial lipopolysaccharide (LPS) was shown to play a role in respiratory syncytial virus (RSV) and mouse mammary tumor virus (MMTV) infections (Rassa et al., 2002; Kurt-Jones et al., 2000; Haynes et al., 2001); and TLR 2 and 9 were implicated in responses to members of the *Herpesviridae* (Compton et al., 2003; Tabeta et al., 2004; Krug et al., 2004; Lund et al., 2003). More recently a TLR-independent pathway involving RIGI/MDA5 for viral recognition has been described (Yoneyama et al., 2004b; Kato et al., 2005). Studies on murine bone-marrow derived DC from control and knockout mice have shown the involvement of TLR2 and TLR9 in pathogenesis and DC inflammatory cytokine production in response to HSV-1 infections (Krug et al., 2004; Kurt-Jones et al., 2004; Hochrein et al., 2004; Mansur et al., 2005; Sato et al., 2006).

Additional PRR are also known to play a role in viral recognition by DC and initiation of an anti-viral response. Studies on HSV-1 infection of Vero cells, HeLa cells and BHK-1 cells suggest that the replication of a virus within these cell results in an accumulation of intracellular double-strandad RNA, which may trigger a host response mechanism (Weber et al., 2006). **Retinoic acid-inducible gene I (RIG-I)** is a highly inducible cytoplasmic member of the DExD/H box-containing RNA helicase family of proteins that signals antiviral responses following the binding of dsRNA (Yoneyama et al., 2004b). When activated, RIG-I triggers the activation of NF-κB and IRF3/7, which in turn induce the production of antiviral type I IFN. This downstream signal is mediated via a recently identified CARD domain containg protein, mitochondrial anti-

viral signaling protein (MAVS) (also known as IPS-1, CARDIF or VISA) (Seth et al., 2005; Kawai et al., 2005; Xu et al., 2005; Sun et al., 2006).

Another DExD/H box RNA helicase family member found to interact with dsRNA is the **melanoma-differentiation-associated gene 5 (MDA5)** which acts in a similar way to RIG-I with some overlapping functions (Yoneyama et al., 2005). Both RIG-I and MDA5 can bind to dsRNA *in vitro*. However, *in vivo* the pathway depends on the virus, with RIG-I responding to Sendai virus, Newcastle disease virus, influenza A and Japanese encephalitis; and MDA5 responds to picornaviruses and to the synthetic dsRNA mimic, poly I:C (Kato et al., 2006).

Another cytosolic receptor that also evokes the nucleic acid-mediated activation of the innate immune response is **DNA-dependent activator of IFN regulatory factors (DAI)**, also known as DLM-1/ZBP1 (Takaoka et al., 2007). Studies on this receptor have shown its direct interaction with DNA to bring about the trigger for type I IFN (Takaoka et al., 2007; Wang et al., 2008). Furthermore, knockout of DAI impairs the induction of type I IFN by dsDNA and HSV-1.

	Location	Ligand(s)
TLR 1	cell surface (in association with TLR2)	Lipoproteins; Triacyl lipopeptides; Pam_3CSK4
TLR 2	cell surface	Bacterial lipoproteins; PG; viral glycoproteins
TLR 3	endosome	Viral dsRNA; poly I:C
TLR 4	cell surface	LPS; Lipid A; MPL; viral glycoproteins; HSP; Fibrinogen
TLR 5	cell surface	Flagellin
TLR 6	cell surface (in association with TLR2)	Diacyl lipopeptides; LTA; Zymosan; Pam_2CSK4
TLR 7	endosome	Viral ssRNA; Imiquimod; R848
TLR 8	endosome	Viral ssRNA; R848; CL075
TLR 9	endosome	Viral DNA; CpG-ODN
TLR 10	cell surface	Unknown

Table 1.5: TLR ligands (adapted from Takeda and Akira, 2007).

1.6.2. DC Subtypes

Using a combination of monoclonal antibodies against cell surface molecules, together with micro-environmental observations, and *in vitro* studies, several different DC subtypes have been described both in humans and in animal models. The DC nomenclature depends on progenitors and precursors, anatomical localization and function, as well as phenotype (Nestle et al., 1993; Takahashi et al., 1998; Banchereau et al., 2000; Bendriss-Vermare N et al., 2001; McIlroy et al., 2001; Summers et al., 2001; MacDonald et al., 2002; Wan H and Dupasquier M, 2005).

Despite extensive research on the development of DC, the differentiation pathways that generate the different DC subtypes *in vivo* are still controversial. There are two current models for DC origin, suggesting the existence of two independent differentiation pathways originating from independent myeloid or lymphoid precursors; and alternatively, the existence of a common DC differentiation pathway that generates all subtypes from a common DC precursor. Based on direct and indirect experimental evidence a schematic diagram of the suggested human DC differentiation pathway is shown in figure 1.5 (Ardavin et al., 2001).

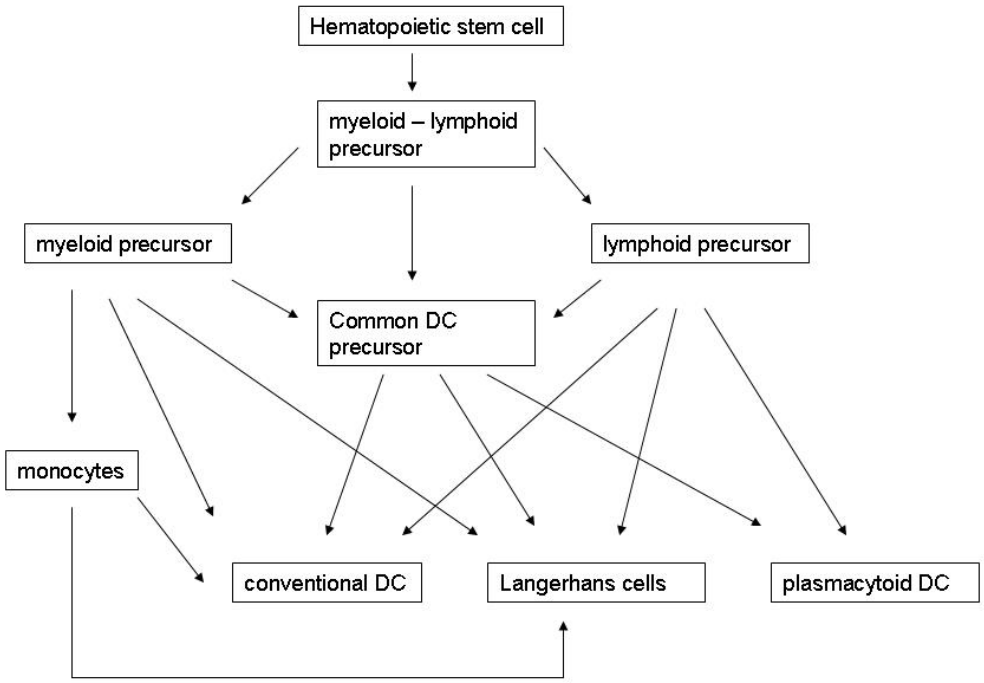


Figure 1.5. Schematic diagram of the proposed origin of human DC subtypes. Two distinct models for the *in vivo* origin of human DC subtypes have been proposed. In the first, two independent differentiation pathways lead to the independent differentiation of conventional DC and Langerhans cells of myeloid origin or conventional DC, Langerhans cells and plasmacytoid DC of lymphoid origin. An alternative model suggests however, the existence of a common DC differentiation pathway that generates all subtypes from one common DC precursor.

As HSV-1 infects predominantly the skin and mucosal surfaces, in this section the main emphasis will be on the two distinct DC subtypes found consistutively in normal skin: epidermal Langerhans cells (LC), and dermal DC (DDC) and on an additional DC subtype not normally present in the skin, but which migrates to the skin following inflammation: plasmacytoid DC (pDC) (fig. 1.6).

Langerhans cells (LC) are a DC subset of the skin, constituting around 2% of the total cellular population of the epidermis (Katz FE et al., 1985). LC have a unique phenotype. They express E-cadherin, a type I transmembrane protein whose role is in cell adhesion to keratinocytes, langerin (CD207), a type II lectin transmembrane cell surface receptor and internal Birbeck granules. Upon antigen uptake, Langerin associates with the Birbeck granules and facilitates the routing from the cell surface into a nonclassical antigen-processing pathway (Valladeau et al., 2000). Interestingly, Langerin knockout mice lack Birbeck granules, but do not demonstrate defective immune responses, suggesting that the Langerin protein and Birbeck granules are non-essential for immunity to skin pathogens (Kissenpfennig et al., 2005). Despite this finding, Langerin and Birbeck granules remain the best marker available for distinguishing LC from other DC subtypes. Furthermore, LC also express low levels of DC-SIGN (Ebner et al., 2004), but lack the macrophage mannose receptor (MMR) (Kato et al., 2000).

The exact role of LC in peripheral immunity remains enigmatic. Early paradigms proposed that these cells signal the presence of antigen or pathogen in the epidermis to the adaptive immune system. According to this model, LC pick up antigen in the epidermis and then emigrate to skin draining lymph nodes (LN) via the dermal

lymphatics (Stoitzner et al., 2002). The presence of cells with dendritic morphology in the afferent lymph, which were presumably representative of this migration, led to the term “veiled cell” (Balfour BM et al., 1981). It was suggested that in the LNs, these cells display a mature phenotype and present the antigens to naive T cells (Stoitzner et al., 2002; Mayerova et al., 2004).

However, this is not the only hypothesis that has been suggested to link peripheral antigen uptake and lymph node antigen presentation. Studies in mouse models did not support this view completely. Freshly isolated LC were not effective APC compared to DC (Inaba K et al., 1986; Katz DR and Sunshine GH, 1986). In experiments where infection was restricted to the mucosal and epidermal epithelia LC were not necessarily directly responsible for *in vivo* activation of specific T cells (Allan et al., 2003; Belz et al., 2004; Zhao et al., 2003; Lemos et al., 2004). On the basis of these studies, it has been suggested that LC play an indirect role, transporting antigen from the epidermis to the LNs, and then transferring the antigens to LN resident DC for antigen presentation and T cell priming.

The origin of epidermal LC is two-fold. Under non-inflammatory conditions, epidermal LCs are renewed locally from a pool of precursors in the skin (Merad et al., 2002). In human studies this was first demonstrated in a study where dermal $CD14^+$ cells were shown to express Langerin and to acquire LC characteristics when cultured with TGF-β (Larregina et al., 2001). This was explored further in a mouse model, where in lethally irradiated mice that had received a bone-marrow transplant from a donor mouse, epidermal LC of host origin could be isolated for at least 18 months post-irradation, while DC in other organs were replaced almost completely within 2 months (Merad et al., 2002). Under inflammation however, following migration of LC into the skin-

draining lymph nodes, these cells are replaced by blood-borne LC progenitors. More recent studies have shown that in the mouse model, these LC precursors are of myeloid orgin (Ginhoux et al., 2006).

TGF-β1 in the epidermal microenvironment is thought to be important for the phenotypic characteristic of LC (Strobl and Knapp, 1999), as TGF-β1 knockout mice are completely devoid of epidermal LC, and these reappear after local injections of recombinant TGF-β1 (Borkowski et al., 1996; Thomas et al., 2001). Because *ex-vivo* purification of LC from human skin brings about a significant spontaneous maturation, possibly due to the cells' dispatch and migration from the skin (Tchou et al., 2003), studies have focused mainly on mouse skin LC and LC-like cells derived from monocytes differentiated in the presence of TGF-β (Geissmann et al., 1998; Geissmann et al., 1999; Nunez et al., 2004).

Dermal DC (DDC) are a subtype of DCs of myeloid origin found within the extracellular matrix of the dermis. DDC can be distinguished from LC by the expression of MMR and DC-SIGN (Turville et al., 2002; Santegoets et al., 2008), as well as the expression of the scavenger receptor CD36 and the coagulation factor XIIIa (Cerio R et al., 1989; Santegoets et al., 2008). In humans these cells lack the characteristic Langerin (CD207) of LC. In the mouse model however, studies have suggested that the dermis and skin draining lymph nodes contain langerin^{low} DC from a non-LC origin as well (Douillard et al., 2005; Ginhoux et al., 2007).

Comparative studies with *in vitro* generated DC derived from CD14^{+} monocytes suggest that the MDDC have a phenotype comparable to that of DDC (Grassi et al., 1998), suggesting a) a myeloid precursor and b) relating DDC to what are commonly known as **conventional DC (cDC)**, the most frequently studied form of DC. Although

originally considered to be of myeloid origin, since they could be produced from both human and mouse bone-marrow myeloid precursors (Reid et al., 1992; Inaba et al., 1993; Caux et al., 1997), more recent studies suggest there may also be some cells that are derived from lymphoid progenitors (Martin et al., 2000; Bell and Bhandoola, 2008; Izon et al., 2001; Manz et al., 2001; Wu et al., 2001; Bell and Bhandoola, 2008). These DC are commonly found in the circulating peripheral blood and lymphoid tissues, as well as the skin, respiratory and gut mucosa, their specific functional capacities depending on the specific tissue.

Unlike DDC which are present in the dermis and express TLR1 – TLR8, ex-vivo epidermal LC express either low levels or undetectable TLR2, TLR4, TLR5 and TLR7 (van der Aar et al., 2007; Takeuchi et al., 2003; Rozis et al., 2000), suggesting that these cells have low reactivity to bacterial pathogens, and thus presumably there is no unnecessary inflammatory response to harmless skin commensals.

Plasmocytoid DC (pDC) are specialized immune cells capable of producing large amounts of type I IFN and other pro-inflammatory cytokines in response to viral infections. In both human and mouse adults, they constitute around 0.2%-0.8% of total peripheral blood mononuclear cells, but are also found in primary and secondary lymphoid tissues, including thymus, tonsil, spleen, lymph nodes and bone marrow (Fitzgerald-Bocarsly and Feng, 2007). pDC are produced continuously in the bone-marrow and circulate in peripheral blood to lymph nodes, mucosal-associated lymphoid tissues and spleen (Cella et al., 1999; Penna et al., 2002; Yoneyama et al., 2004a). Studies on pDC development suggest that Flt3L is the main cytokine driving their differentiation from haematopoetic stem cells (Pulendran et al., 1998; Blom et al., 2000; Brawand et al., 2002; Gilliet et al., 2002; Chen et al., 2004). Unlike MDDC (which *in*

vitro resemble DDC) that display TLR1-6, TLR10 and low levels of TLR7, and LC, which express either low levels or no TLR2, TLR4, TLR5 and TLR7 (Rozis et al., 2000; Kadowaki et al., 2001; van der Aar et al., 2007), pDC lack TLR2, TLR3 TLR4, TLR5, TLR6 and TLR8 completely, but do express the endosomal TLR7 and TLR9 responsible for DNA and RNA virus recognition (Iwasaki and Medzhitov, 2004). Although activated pDC have been shown to present antigens and induce expansion of T cell population, albeit less efficiently than DC (Krug et al., 2003; Fonteneau et al., 2003; Schlecht et al., 2004) their main function is in the early production of large amounts of type I IFN (Dalod et al., 2003).

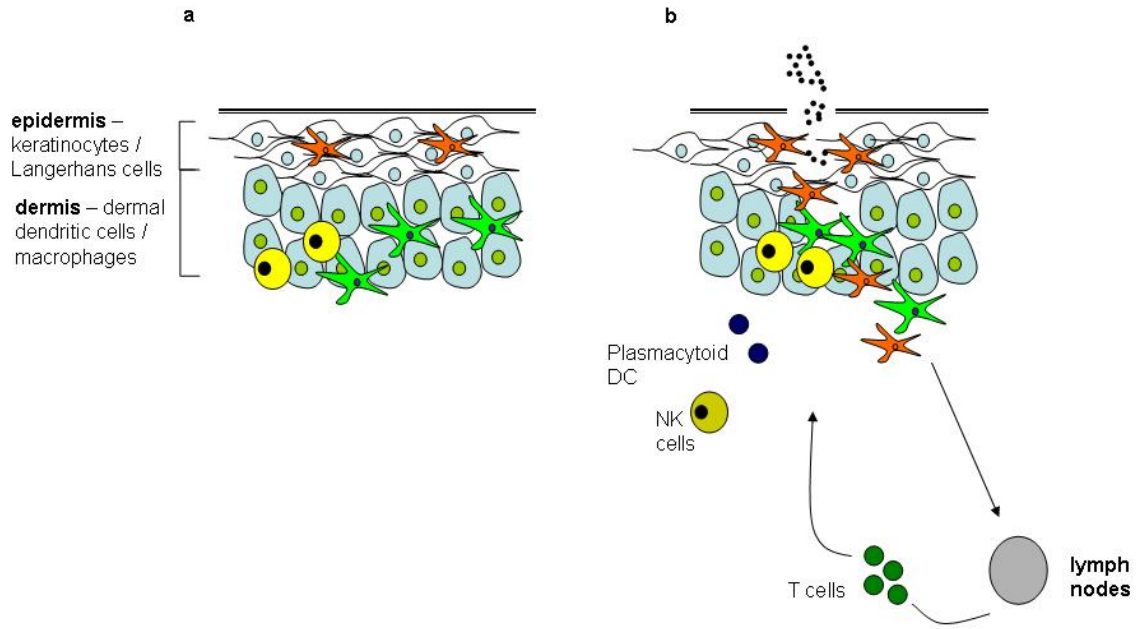


Figure 1.6: Schematic diagram of primary HSV-1 infection of the skin. The skin is the first line of defence against infection. The barrier function has two associated immune components: innate-inflammatory which involves the recognition of microbial compounds and the subsequent expression of pro-inflammatory cytokines and interferons; and the "early warning" system which attracts the adaptive immune response. a) In non-infected normal skin, the epidermis, made up of resident keratinocytes and LC, and the dermis, consisting of resident DDC and macrophages within an extracellular matrix serves as a barrier, the cells sampling for foreign antigens. b) In the presence of invasion by an infectious agent, these cells capture the foreign antigen releasing non-specific pro-inflammatory cytokines, and recruit more cells to site of infection, including pDC and NK cells. Upon activation, DC and macrophages migrate to the lymph nodes where they interact with other resident DC and naïve T and B cells to initiate the adaptive immune response.

1.7. Hypothesis, aims and objectives of the thesis

In this introduction, the biology of HSV-1 and aspects of the immune response against it, specifically the role of DCs has been discussed. Previous studies from this laboratory have shown that wild-type HSV-1 infects DC readily leading to maturation as judged by surface phenotype. Infected DC produced IFN-α, yet lose their antigen presenting abilities. This was shown to be dependent on viral gene expression, as UV inactivated virus was shown to mature and activate DC (Pollara et al., 2004a), suggesting a role for the initial interaction between the viral surface and the cellular membrane in DC maturation.

The aim of the work reported in this thesis is to investigate the hypothesis that the HSV-1 glycoproteins necessary for viral entry into cells also play a role in DC activation, and therefore can play a role in the initiaition of an immune response.

This hypothesis will be investigated by exploring three main objectives, each of which is reported and discussed in a separate chapter:

1. The development of an *in vitro* model in which individual HSV-1 glycoproteins are expressed independent from the rest of the virus and used to study the collaborative interaction between individual HSV-1 glycoproteins and MDDC.

2. The investigation of the biological consequences of the HSV-1 glycoprotein-DC interaction as evidenced by the ability of the DC to activate T cells, to influence the signaling pathways that lead to cytokine secretion, and synthesize and release cytokines.

3. The comperative investigation of the interaction of the HSV-1 glycoproteins with other DC subtypes, specifically LC and pDC.

CHAPTER 2 - MATERIALS AND METHODS

2.1. Safety and Sterile Conditions

All tissue culture work was performed in a class II safety cabinet, using sterile techniques.

2.2. Primary cells

Unless otherwise stated, all short and long term cell culture incubations were carried out at 37ºC with 5% CO_2.

2.2.1. Monocyte-derived dendritic cells (MDDC) preparation

MDDC were prepared from 120 ml fresh whole blood from healthy volunteers. The study was approved by the joint University College London/University College London Hospitals NHS Trust Human Research Ethics Committee (Ethics Committee Reference BRD/06/132) and written informed consent was obtained from all participants. Mononuclear cells separated on lymphoprep (Nycomed Pharma) (400 g, 30 mins, no brake), were incubated in six-well tissue culture plates in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (v/v), 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (Clare Hall Laboratories, Cancer Research U.K.) as complete medium. After two hours incubation non-adherent cells were removed and the adherent cells were cultured in fresh complete medium with 100 ng/ml human recombinant granulocyte – macrophage colony stimulating factor (GM-CSF) and 50 ng/ml IL-4. After a four day incubation, loosely adherent cells were collected and any remaining lymphocytes depleted by incubation with mouse anti-human monoclonal antibodies (mAb) against CD2 (to remove T cells and NK cells), CD3 (to remove T cells), and CD19 (to remove B cells), followed by anti-mouse IgG-coated immunomagnetic Dynabeads (Dynal Biotech, Merseyside, U.K.). The DCs were cultured for a further three days in complete medium with fresh

GM-CSF and IL-4 at a concentration of 5 x 10^5 DC/ml and then used as immature DC for stimulation. Throughout the thesis these cells will be referred to as MDDC.

2.2.2. Monocyte-derived Langerhans cells (MDLC)

MDLC were prepared similarly to MDDC, but with an addition of 10 ng/ml TGF-β (R&D systems) on day 0 and day 4 of culture. These cells will be referred to as MDLC.

2.2.3. Plasmacytoid dendritic cells (pDC)

pDC were isolated from peripheral blood mononuclear cells (PBMC) by immunomagnetic cell sorting (MACS CD304 microbead separation kit, Miltenyi Biotec). 120 ml fresh peripheral blood was separated on lymphoprep (Nycomed Pharma) (400 g, 30 mins, no brake) and resuspended in MACS buffer (HBSS with 0.5% bovine serum albumin (BSA) and 2mM EDTA). pDC were then labled with microbeads conjugated to anti-CD304 (BDCA-4/Neuropilin) mouse monoclonal antibodies (Miltenyi Biotec) (4ºC, 15 mins) and passed twice through a magnetic separation column (MS column, Miltenyi Biotec). pDC were counted and viability determined using trypan blue, before culture in complete medium.

2.2.4. Epidermal Langerhans cells (LC)

Human dermatome sections of skin were recovered from mammoplasty surgery with informed consent and local ethical approval from Newcastle and North Tyneside Local Research Ethics Committee. Epidermal LC were a gift from M.P. Collin (University of Newcastle upon Tyne) and were isolated at the University of Newcastle upon Tyne as described previously (Collin MP et al., 2005). In brief, skin sheets were incubated in RPMI supplemented with Dispase 1mg/ml (Invitrogen) for 60 mins at 37ºC. Epidermal sheets were then peeled from the dermis and rinsed in PBS. Fresh LC were isolated by

incubating the epidermal sheets in 0.25% trypsin for 10 mins at room temperature. Migratory LC were isolated by floating epidermal sheets for 72 hrs in complete RPMI medium supplemented with GM-CSF.

2.2.5. DC stimulation

Day 7 purified immature MDDC / MDLC ($2x10^5$ – $5x10^5$) were stimulated with Pam3Cys (100ng/ml; InvivoGen), polyinosine-polycytidylic acid (poly I:C) (50μg/ml; InvivoGen), lipopolysaccharide (LPS) (100ng/ml; *E. coli* 0111:B4, InvivoGen), imiquimod (1mg/ml; InvivoGen), CL075 (1mg/ml; InvivoGen); CpG oligodeoxynucleotides (5μg/ml; Coley) or recombinant human soluble CD40 ligand (1μg/ml; PeproTech). In most experiments known concentration of LPS were used as a control. .

2.2.6. Preparation of T cells

T cells were obtained from the non-adherent population of PBMC which was described in section 2.2.1. Collected cells were resuspended at approximately $5x10^6$ cells/ml and cyropreserved in FCS containing 10% dimethyl sulfoxide (DMSO) (v/v). Samples were frozen slowly to -70ºC before transfer to liquid nitrogen for long term storage.

When required, cells were thawed rapidly (37ºC water bath) and activated T cells, B cells, monocytes, and macrophages were depleted by incubation with CD19, HLA-DR, and CD14 mAb for 30 mins on ice. Cells were washed and then mixed with anti-mouse IgG-coated immunomagnetic Dynabeads (Dynal Biotech) and separated on magnetic columns. These T cells were used immediately after purification.

2.3. Cell and Tissue Culture

Similar to primary cells, all short and long term cell culture incubations were carried out at 37ºC with 5% CO_2.

2.3.1. Cell lines

COS-7

The Cos-7 African green monkey kidney fibroblast-like cell line is derived from CV-1 simian cells transformed by an origin-defective mutant of SV40. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (PAA Laboratories, Linz, Austria) (v/v) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich) (complete DMEM).

Cos-7 cells were cultured until 80-90% monolayer confluency after which the cells were washed once with Hank's Buffered Salt Solution (HBSS), detached from the surface of the flask with 2mM ethylene diamine tetra-acetic acid (EDTA) and re-cultured at ratio 1:5 in complete DMEM.

BHK

The baby hamster (Golden Syrian) kidney 21, clone 13, fibroblast-like cell line was grown and cultured similarly to Cos-7.

CHO

The Chinese hamster ovary epithelial-like cell line was grown and cultured similarly to Cos-7.

HaCaT

The Human Keratinocyte Cell Line was grown and cultured similarly to Cos-7.

2.3.2. Storage of Cell Lines

For long term storage, cells were centrifuged at 300 g for 5 minutes and resuspended in 90% fetal calf serum (FCS) and 10% DMSO (v/v). Freezing vials were slowly cooled to -70ºC before transferred to liquid nitrogen for long term storage. For recovery, cells were thawed rapidly in 37ºC water bath, diluted in HBSS and centrifuged at 300 g, 5 minutes. The pellet was then resuspended in complete medium and transferred into a 25cm^2 flask for culture.

2.3.3. Transfection

Cos-7 and CHO cells were cultured for 24 hrs before being transfected transiently using FuGENE 6 transfection reagent (Roche Diagnostics) with vectors expressing HSV wild-type glycoproteins or mutant glycoproteins (table 2.3). Cells were cultured for a further 48 hours to allow glycoprotein expression at the cell surface. Glycoprotein expression was verified by flow cytometry and confocal microscopy using glycoprotein-specific mAbs.

Transfected cells were used to stimulate DC by over-layering DC on to the cells in complete medium at a ratio of 1:10 (discussed in detail in section 3.2.3).

2.4. Virus preparation

2.4.1. Virus Growth

The Herpes Simplex Virus (HSV)-1 construct used was derived from strain 17+ containing a cassette of a cytomegalovirus (CMV) immediate early (IE) promoter that

drives expression of green fluorescent protein (GFP; Clontech) and a respiratory syncytial virus promoter that drives expression of β-galactosidase. This cassette is inserted into the UL43 gene, as described elsewhere (Coffin et al., 1996). This virus was a gift from R.S. Coffin (Biovex).

The virus was propagated on confluent BHK in complete DMEM. 48 hours post infection, cellular debris was removed by low speed centrifugation (3000 g, 30 mins, 4°C). The supernatant was removed immediately and filtered through a double filter (0.65-µm and 0.45-µm, respectively) before being centrifuged at 16000 g for 2 hrs at 4°C. The supernatant was discarded and the viral pellet was resuspended gently in 1 ml complete DMEM. The prepared aliquots were stored at –70°C until use. This virus preparation is referred to as HSV/GFP.

2.4.2. Viral infectivity assay

Viral infectivity and titre was determined by plaque assay on BHK cells. Serial dilutions [(10^4 to 10^9 plaque forming unit (pfu)] of virus in complete DMEM was added to a confluent BHK monolayer in a 24 well plate in quadruplicate. After 30 mins, incubation, 1ml of 1:3 (v/v) of 0.5% carboxymethyl cellulose : complete DMEM was added to each well. Cells were incubated for 48 hours and the number of plaques in each well were counted using fluorescence microscopy in order to determine virus titre in pfu/ml.

The virus stock used in this entire study had a titer of 1 x 10^9 pfu/ml.

2.4.3. Viral inactivation

HSV/GFP fixed virus was inactivated by incubating virus in 0.05% glutaraldehyde (Sigma-Aldrich) in PBS for 2 mins. Quenching of excess glutaraldehyde was performed

by diluting the virus in complete DMEM before adding to the cells. This virus is referred to as Fix-HSV/GFP.

Neutralization of viral glycoproteins was performed using mouse monoclonal antibodies to HSV gD (LP2 and AP7), heterodimer gHgL (LP11) and gB (2153) (all gifts of Dr. H. Browne, University of Cambridge, UK). Virus was incubated with specific mAb for 30 mins on ice before used to infect cells.

2.4.4. Viral infection of DC

Immature day 7 MDDC / MDLC were infected with HSV/GFP. GFP expression upon infection was determined by fluorescence microscopy or flow cytometry 18-24 hours post infection.

2.5. Flow cytometry

All stained cells were acquired on a FACScan or FACS Calibur flow cytometer using CellQuest software (BD Biosciences). For each sample at least 5000 events were acquired. Data was later analyzed with WinMDI v2.8 software (Joseph Trotter, Scripps Research Institute).

2.5.1. Cell Surface Immunofluoresence

For cell surface immunofluoresence, cells were harvested 18-24 hours post-stimulation. Non-specific antibody binding was blocked with 10% goat serum or 10% rabbit serum (20 mins, 4°C), depending on the secondary antibody. DC were then stained for specific surface markers by incubation first with the relevant mAb (30 min, 4°C) followed by 1:50 diluted phycoerythrin (PE)-conjugated goat anti-mouse Ig or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (both from Dako) (30 min, 4°C).

For double staining, after removal of secondary conjugate cells were incubated in 1% mouse serum (10 mins, 4°C) and then further stained with directly-conjugated mAb (20mins, 4°C). Cells were washed and fixed in HBSS containing 3.7% paraformaldehyde (v/v). Samples were stored at 4°C in the dark and analyzed within 24 hours.

2.5.2. Intracellular Immunofluoresence

For intracellular immunofluoresence staining, cells were harvested 1 hour or 5 hours post-stimulation. Cells were immediately fixed in tris buffered saline (TBS) containing 3.7% paraformaldehyde (v/v) for 15 minutes. After washing off the fixative, non-specific antibody binding was blocked with 10% goat serum (20 mins, 4°C). Cells were then stained for specific surface markers by incubation with directly-conjugated mAb (20 mins, 4°C), before permeablization with 90% methanol (10 minutes). After washing, non-spesific antibody binding was again blocked with 10% bovine serum albumin (BSA), and the cells stained by incubation with directly-conjugated mAb (20 mins, 4ºC). Cells were washed and resuspended in TBS. Samples were stored at 4°C in the dark and analyzed within 24 hours.

2.6. T cell proliferation assay

All proliferation assays were performed using allogeneic T cells from HLA-mismatched donors and purified as described in section 2.2.5. Titrations of purified DC were incubated with 1 x 10^5 allogeneic T cells in flat-bottomed 96-well microtiter plates for 5 days and then pulsed with 1 μCi of [^{3}H] thymidine (ICN Biomedical) for the final 18 h of culture. All proliferation assays were performed in triplicate wells. Cells were harvested, and T-cell proliferation was measured by liquid scintillation counting (Microbeta Systems). Results were expressed as counts per minute (cpm).

2.7. ELISA

After 18-24 hour stimulation, supernatant was harvested and used to assay for DC cytokine secretion. IFNα was measured in cell supernatant using ELISA according to the company protocol (PBL Biomedical). Sandwich ELISA was set up for the detection of IL-12p70, IL-10 and TNF-α. ELISA plates were coated overnight with 1μg/ml IL-12p70 (clone BT-21), IL-10 (clone JES3-9D7) or TNF-α (clone MAb1) (all from eBioscience) or 4μg/ml IL-8 (clone MAB208, R&D) capture antibodies in PBS at 4ºC. The plates were blocked with 1% BSA in PBS for 2 hours at room temperature (RT). After washing with PBS containing 0.05% Tween-20 (PBS-Tween), the plates were incubated with 50 μl sample for 60 minutes at RT. Standards (eBioscience) were diluted in complete medium. The plates were washed in PBS-Tween and incubated with 1 μg/ml biotin anti-human IL-12 (clone C8.6), anti-human IL-10 (clone JES3-12G8) or anti-human TNF-α (MAb11) (all from eBioscience) or 20 ng/ml IL-8 (clone BAF208, R&D) detection antibody in PBS-Tween for 60 minutes at RT. Next, the plates were again washed with PBS-Tween and incubated with 50 μl streptavidin horseradish peroxidase (HRP)-conjugated (R&D Systems) in PBS-Tween for 30 min at RT. After further washing, HRP presence was detected using tetramethlybenzidine (TMB) substrate (eBioscience). Colorimetric reactions (450nm) were quantified immediately on the MRX Revelation plate reader using the Revelation v4.21 software (Dynex Technology) for analysis. All concentrations were determined in accordance with the standards (fig. 2.1).

For T cell cytokine secretion, supernatants were collected 24 hours and 6 days post T cell-DC co-culture. Sandwich ELISA was set up for the detection of IFN-γ (capture: clone MD-1, 4 μg/ml; detection: clone 4S.B3, 1 μg/ml) IL-13 (capture: clone PVM13-1,

2 μg/ml; detection: clone polyclonal, 1 μg/ml) and IL-17A (capture: clone eBio64CAP17, 1 μg/ml; detection: eBio64DEC17, 1 μg/ml) as described above.

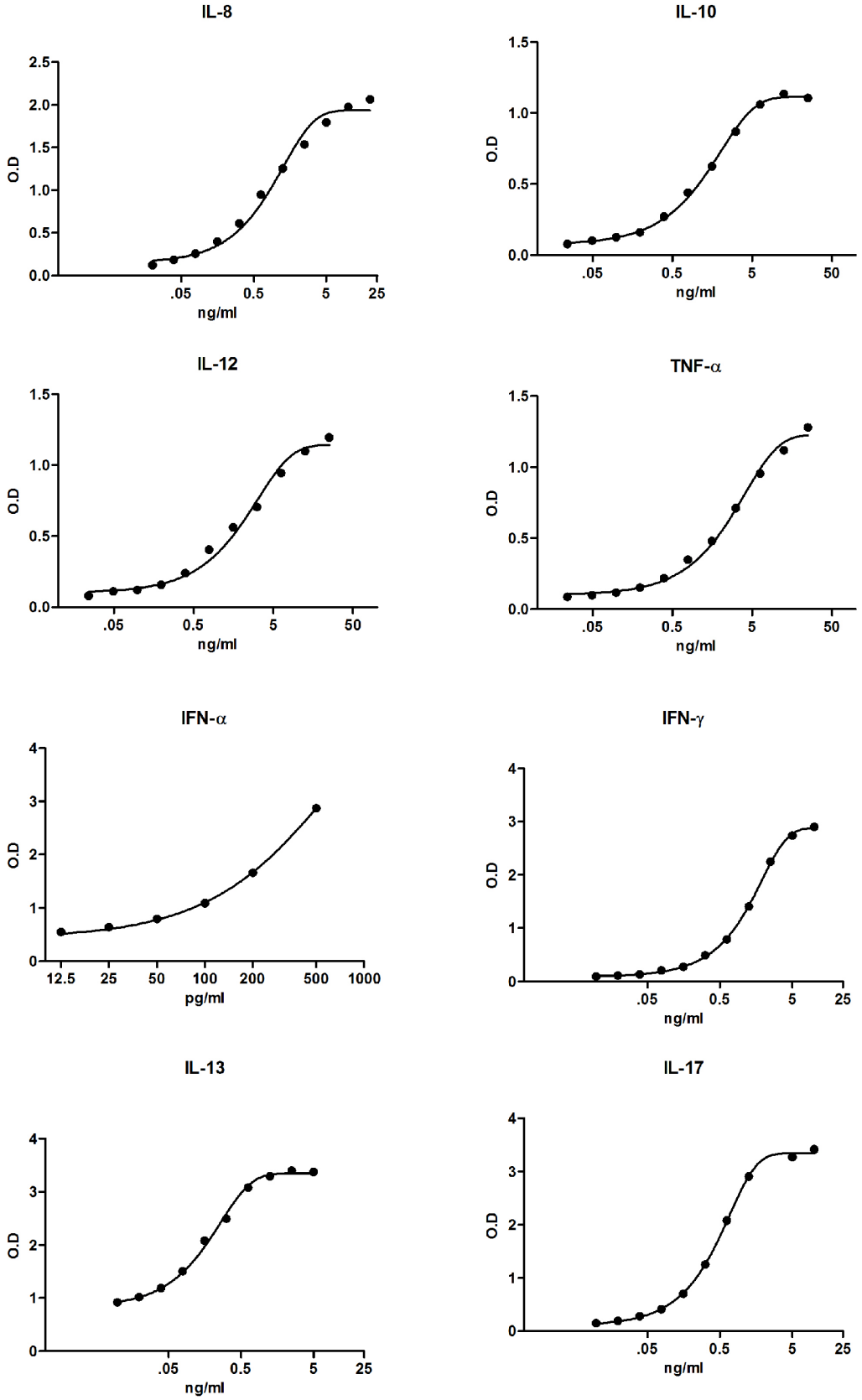


Figure 2.1: ELISA standard curves.

2.8. Quantitative real-time PCR

Taqman qPCR was used for the detection and quantification of IFN-β gene expression. Total RNA was isolated using RNeasy Mini Kits (Qiagen) from MDDC or MDLC activated with LPS (100 ng/ml), poly I:C (50 μg/ml), or infected with HSV (MOI 1), or cells co-cultured with Cos-7 cells expressing gBgD/gHgL, or mock transfected. First strand cDNA was synthesized from the purified RNA using the first strand cDNA synthesis kit according to manufacturer's instructions (New England Biolabs). The following primers and probe were used for the detection of IFN-β: 5'-GAGCTACAACTTGCTTGGATTCC, 3'-CGTTAACTTACCCTCCGAAC and probe 5'-ACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCT with 5' 6FAM fluorescent dye and 3' BHQ1 quencher. For each sample, the quantitative results were normalized to the quantitative levels of the "housekeeping" glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA using the primers: 5'-GGCTGAGAACGGGAAGCTT, 3'-AAGGTCCTCGCTCTAGGGA and probe 5'-TCATCAATGGAAATCCCATCACCA. For all reactions, the cDNA were first denatured for 5 minutes at 95°C and then subjected to 50 cycles of PCR amplification of 15 seconds at 95°C, followed by 50 seconds at 60°C. Real time PCR amplification was quantified with the ABI Prism 7000 System (PE Applied Biosystems).

2.9. Microscopy

All confocal images were obtained with a Leica SP2 confocal microscope (Leica Confocal microscope) with a pin hole of 1 Airy (114.5 μm), scan speed of 400 Hz and 4 frame averaging. The images were analyzed using the Leica Confocal software.

2.9.1. Confocal microscopy – cell surface staining

For cell surface staining for confocal microscopy, cells cultured on glass coverslips were harvested 18-24 hours post-stimulation and fixed with 3.7% paraformaldehyde for 15 minutes prior to incubation with antibodies. Non-specific antibody binding was blocked with 10% goat serum (30 mins, RT). Cells were stained for specific surface markers by incubation first with the relevant mAb (1 hour, RT) followed by 1:100 diluted secondary conjugate goat anti-mouse Ig (Dako) (1 hour, RT). Cells were washed and stained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI) (2μg/ml) for 5 minutes and mounted on slides. Samples were stored at 4°C in the dark until analysis.

2.9.2. Confocal microscopy – intracellular staining

For intracellular staining for confocal microscopy, cells cultured on glass coverslips were harvested 1-6 hours post-stimulation and fixed with 3.7% paraformaldehyde. After washing off the fixative, cells were permeablized with TBS containing 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes, non-specific antibody binding was blocked with 10% goat serum, and the cells stained by overnight incubation (4ºC) with primary antibody. Cells were then washed and stained with secondary conjugated goat anti-rabbit Ig and nuclear counterstaining was performed with the DNA stain DAPI (2μg/ml) and mounted on slides. Samples were stored at 4°C in the dark until analysis. Image analysis was performed with Metamorph v7.17 (Molecular Devices) to quantify nuclear: cytoplasmic ratios.

2.9.3. Electron microscopy

For electron microscopy, all cells were grown on 6mm Thermonox coverslips and fixed in glutaraldehyde at 4ºC, overnight.

Scanning EM

Coverslips were washed in PBS before fixed with osmium tetroxide solution for 30 minutes. Coverslips were then washed in dH_2O and dehydrated through graded acetone 30%, 50%, 70%, 90% and 100%. Tetramethylsilane was then added, and the coverslips were air-dried. SEM images obtained using Philips 501 with Orion digital camera attachment.

Transmission EM

Coverslips were cut into strips, placed in screw cap and washed in dH_2O. Coverslips were then fixed in osmium tetroxide solution to preserve phospholipoproteins, washed and dehydrated through graded alcohols 30%, 50%, 70%, 90% and 100%. Cells were then infiltrated with 1:1 resin:alcohol mixture, followed by 100% resin infiltration and embedded in labelled embedding moulds. Resin was removed from the non-cell side of coverslips and plastic was stripped from the cells. The resin containing cells was cut into semi-thin and ultra-thin sections using a Reichert Ultracut E. TEM images obtained using Philips CM120 (FEI) with AMT 2k digital camera attached.

2.10. Analysis of TLR activation

Engineered HEK293 cells stabely transfected with a plasmid that constitutively expresses a single human TLR gene (Invivogen and gift of N. Witt) were used to test for viral triggering of the TLRs. Cells were grown in complete DMEM supplemented with 100 μg/ml Normocin and 10mg/ml Blasticidin or 10mg/ml Puromycin. At 80% confluency, cells were removed and seeded at 5x104 cells / well in a 96 well plate. Cells were stimulated for overnight incubation (37ºC, 5% CO2) and the supernatant analyzed for IL-8 production.

2.11. Statistical analysis

Where appropriate, the means of paired groups were statistically analyzed using a 2-tailed Student's *t*-test or the one-way Anova test.

List of antibodies

Antibody	Clone	Source
CD1a	NA1/34 (supernatant)	Gift of A. McMichael
CD2	RPA-2.10 (purified)	eBioscience
CD3	UCHT1 (supernatant)	Gift of P.C.L. Beverley
CD14	HB246 (supernatant)	Gift of P.C.L. Beverley
CD19	BU12 (supernatant)	Gift of D. Hardie
CD40	5C3 (purified)	eBioscience
CD80	2D10.4 (purified)	eBioscience
CD83	HB15e (purified)	eBioscience
CD86	BU63 (supernatant)	Gift of D. Hardie
HLA-DR	L243 (supernatant)	Gift of P.C.L. Beverley
Mouse IgG1	Culture supernatant	Dako
Mouse IgG2a	Culture supernatant	Dako
CCR7	150503 (purified)	R&D Systems
E-cadherin	67A4 (purified)	Santa Cruz Biotechnology
NF-kB p65	C-20 (purified)	Santa Cruz Biotechnology
IRF3	FL-425 (purified)	Santa Cruz Biotechnology
IRF7	H-246 (purified)	Santa Cruz Biotechnology
HVEM	Rabbit antiserum	Gift of P. Spear
Nectin-1	CK-41 (purified)	Gift of C. Krummenacher

Table 2.1: Non-conjugated antibodies to human antigens

Antibody	Clone	Source
CD13-APC	WM15	BD Bioscience
CD13-PE	WM15	BD Bioscience
CD209 (DC-SIGN)-PE	DCN46	BD Bioscience
CD303 (BDCA-2)-APC	AC144	Miltenyi Biotec
p-NF-kB p65 (pS529)	K10-895.12.50	BD Bioscience

Table 2.2: Conjugated antibodies to human antigens

Glycoprotein	Backbone	Source
gBgD	pcDNA3.1	Gift of A. Minson
gHgL	pcDNA3.1	Gift of A. Minson
gB (pSR175)	pCMV3	Gift of G.H. Cohen and R.J. Eisenberg
gD (pDC390)	pcDNA3.1	Gift of G.H. Cohen and R.J. Eisenberg
gH (pHC138)	pCMV3	Gift of G.H. Cohen and R.J. Eisenberg
gL (pCMV3gL-1)	pCMV3	Gift of G.H. Cohen and R.J. Eisenberg
gH σLeu (L382P,GLL384-386WPP)	pcDNA3.1	Gift of G. Campadelli-Fiume
gH HR1-EL (E450G,L453A)	pcDNA3.1	Gift of G. Campadelli-Fiume
gH HR2-down2 (LARA570-573AVPQ)	pcDNA3.1	Gift of G. Campadelli-Fiume
gD- pDL485 (37C-302C)	pcDNA3.1	Gift of G.H. Cohen and R.J. Eisenberg
gD- pDL449 (D30A)	pcDNA3.1	Gift of G.H. Cohen and R.J. Eisenberg
gD- pDL490 (3C-38C)	pcDNA3.1	Gift of G.H. Cohen and R.J. Eisenberg

Table 2.3: HSV-1 vectors

CHAPTER 3 - HSV-1 ENTRY GLYCOPROTEIN MEDIATED MATURATION OF MDDC

3.1. Introduction

Infection of DC by HSV-1 leads to a series of both morphological and functional changes which result in partial DC maturation, impairment of antigen presenting function and eventual apoptosis (Kruse et al., 2000; Pollara et al., 2003; Salio et al., 1999). The relationship between viral attachment, fusion and entry, and the consequent signals which result in DC activation remain obscure. Inhibition of DC function is dependent on viral gene expression, since ultra violet (UV) light-inactivated virus, and mutants which do not express essential early transactivators, do not show these inhibitory effects (Pollara et al., 2003; Samady et al., 2003). In contrast, DC maturation is induced by UV treated or formaldehyde-inactivated HSV-1 particles (Pollara et al., 2003). Hence, a role for the viral structural proteins in the activation of DC has been suggested.

As outlined in chapter 1, four viral surface glycoproteins - gB, gD, gH and gL, are necessary for viral entry into cells. Significantly, these same four entry glycoproteins individually also play a key role in the interaction between HSV and the host immune system as they are important targets of adaptive immunity. However, their key role in initiating the early innate immune response to HSV is not well understood. Understanding the complex functions of these HSV proteins will facilitate in our understanding of HSV-1 pathogenesis, but also in the successful development of vaccines.

3.2. Results

3.2.1. Monocyte-derived dendritic cell phenotype

MDDC were prepared as described in section 2.2.1. Consistent with the MDDC phenotype (Pickl et al., 1996), day 7 DC cultures were a homogenous population of

cells expressing high levels of CD1a and CD13 (fig. 3.1). To confirm the purity of the population, and absence of "contamination" from other non-DC PBMC, the cells were stained for the expression of CD3, CD14 and CD19 (T cells, monocytes and B cells respectively). As expected, the DC culture contained less than 1% CD19+ B lymphocytes or CD3+ T lymphocytes. These cells expressed low levels of CD14, consistent with the MDDC phenotype (fig. 3.1b). Fig 3.1c shows representative transmission and scanning EM images of DCs. The transmission image morphology shows a rounded mononuclear cell with a prominent nucleolus. The most striking feature are the numerous long processes which envelope the cell. These are relatively organelle free. There is little active secretory material and few lysosomes in the cytoplasm. The features resemble those of "veiled" cells. Scanning EM shows an elongated cell from which several dendritic processes are extended. In addition there are many surface fine processes. The features resemble those seen in our previous studies of these cells (Swetman CA et al., 2002).

One important characteristic of DC is the ability to transform from an immature state of antigen capture to a mature state of antigen presentation upon a signal from a pathogen – either by direct recognition, or indirect sensing of infection via soluble mediators. This transformation was confirmed phenotypically (fig. 3.2a) and morphologically (fig. 3.2b). MDDC showed upregulation of surface markers CD40, CD83 and CD86, that are associated with this maturation, and of MHC class II (HLA-DR), upon stimulation with lipopolysaccahride (LPS). Thus both a) the immaturity state of day 7 cultured MDDC and b) their ability to acquire the surface phenotype of potent APCs was demonstrated clearly.

3.2.2. MDDC susceptibility to HSV-1

As described previously, DCs are infected readily by HSV (fig. 3.3b). Using HSV/GFP as described previously, dose dependent infection could be monitored by measuring expression of GFP by flow cytometry (fig. 3.3a). Infection of DC at an increasing range of multiplicity of infection (MOI) resulted in overall high infection efficiency, and this increase was dose dependent.

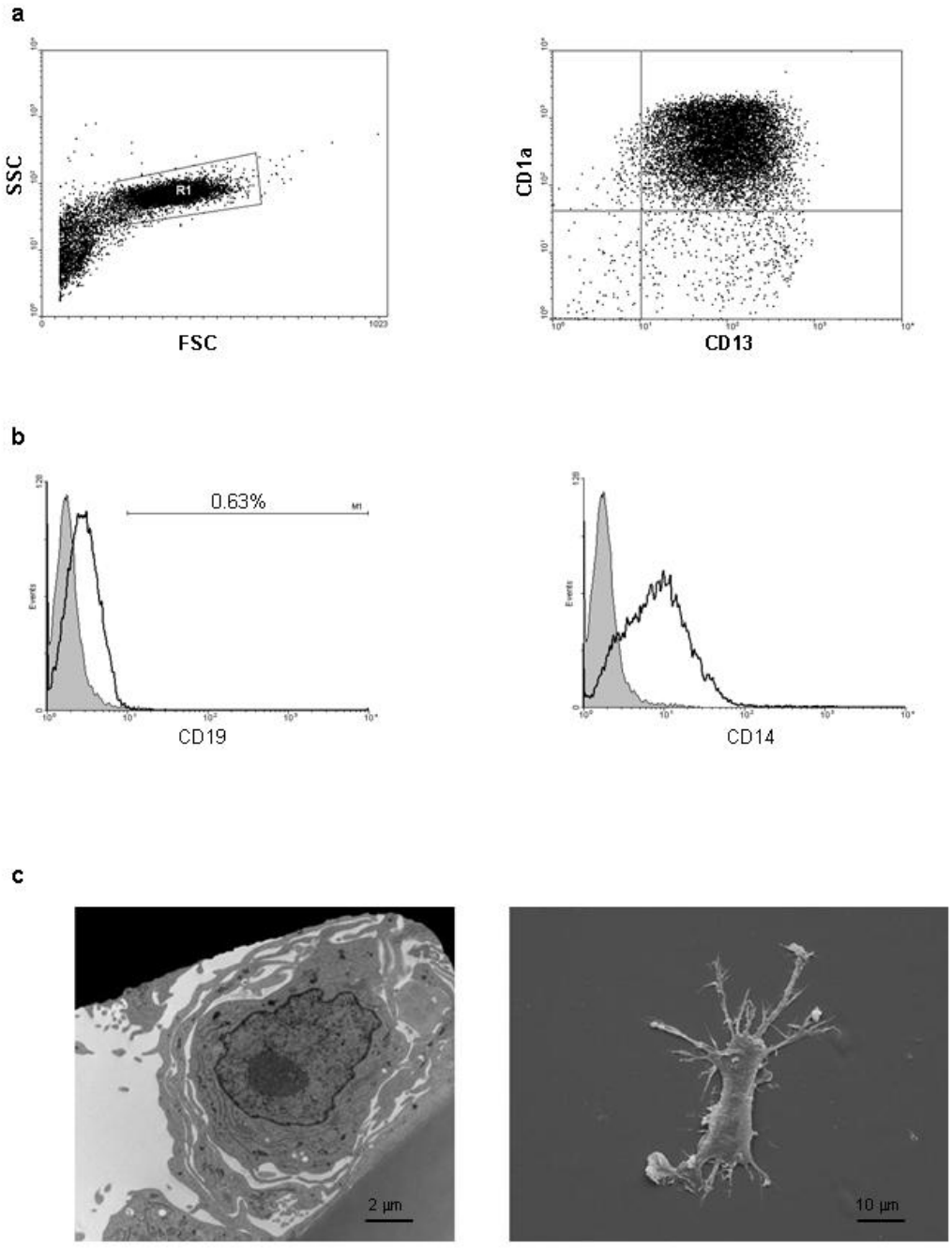


Figure 3.1: Phenotype of immature DC. MDDC were prepared as described in Materials and Methods and analysed by flow cytometry. a) A representative FSC/SSC profile is shown in the left panel. Subsequent analysis is gated on the major live cell population shown as R1. R1 cells almost all expressed both the myeloid marker CD13 and the MDDC marker CD1a. b) R1 cells contained few B cells (stained with CD19) and express low levels of CD14. Filled profile shows staining with IgG control. c) Transmission and scanning EM of unstimulated MDDC.

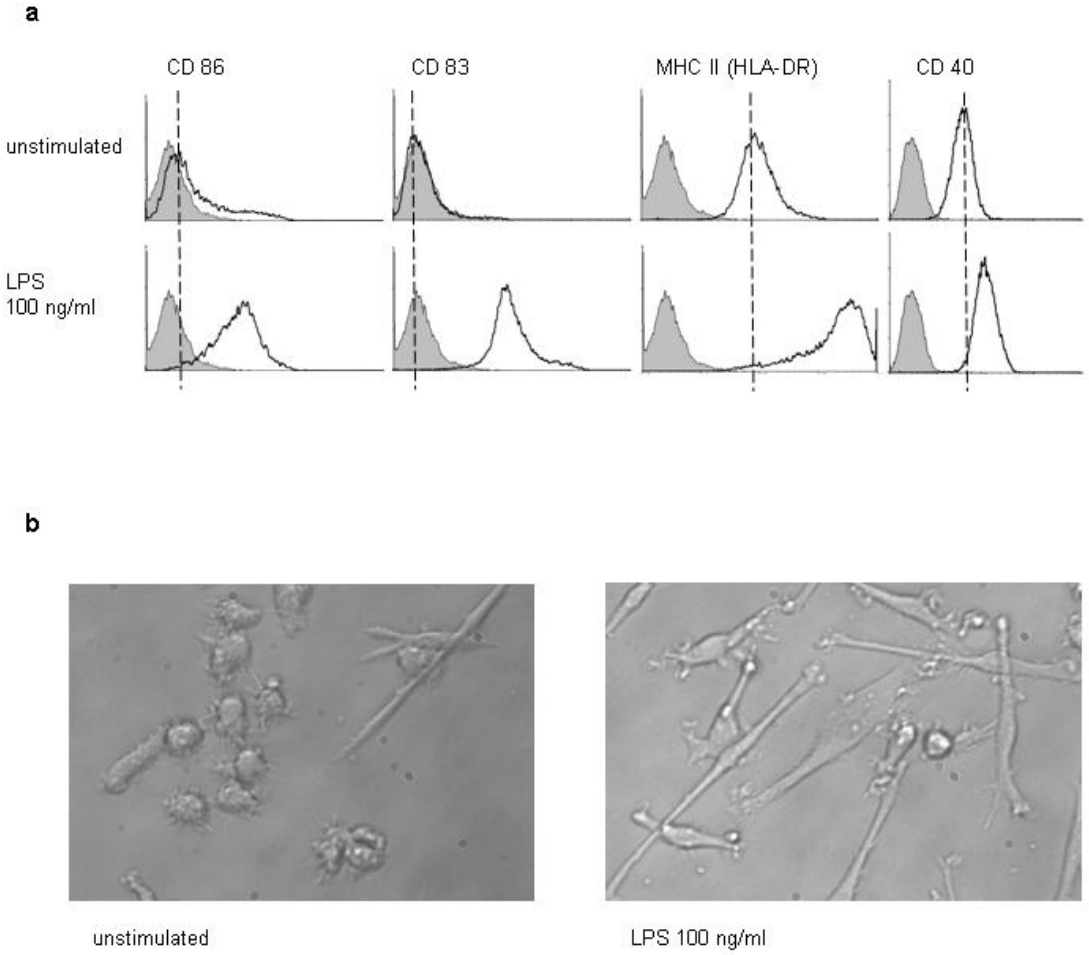


Figure 3.2: Maturation of MDDC. a) Representative example of MDDC stimulated with 100 ng/ml LPS showing upregulation of maturation markers CD86, CD83 and CD40, and also of HLA-DR. b) Morphological changes seen following MDDC maturation as captured by inverted phase contrast microscopy.

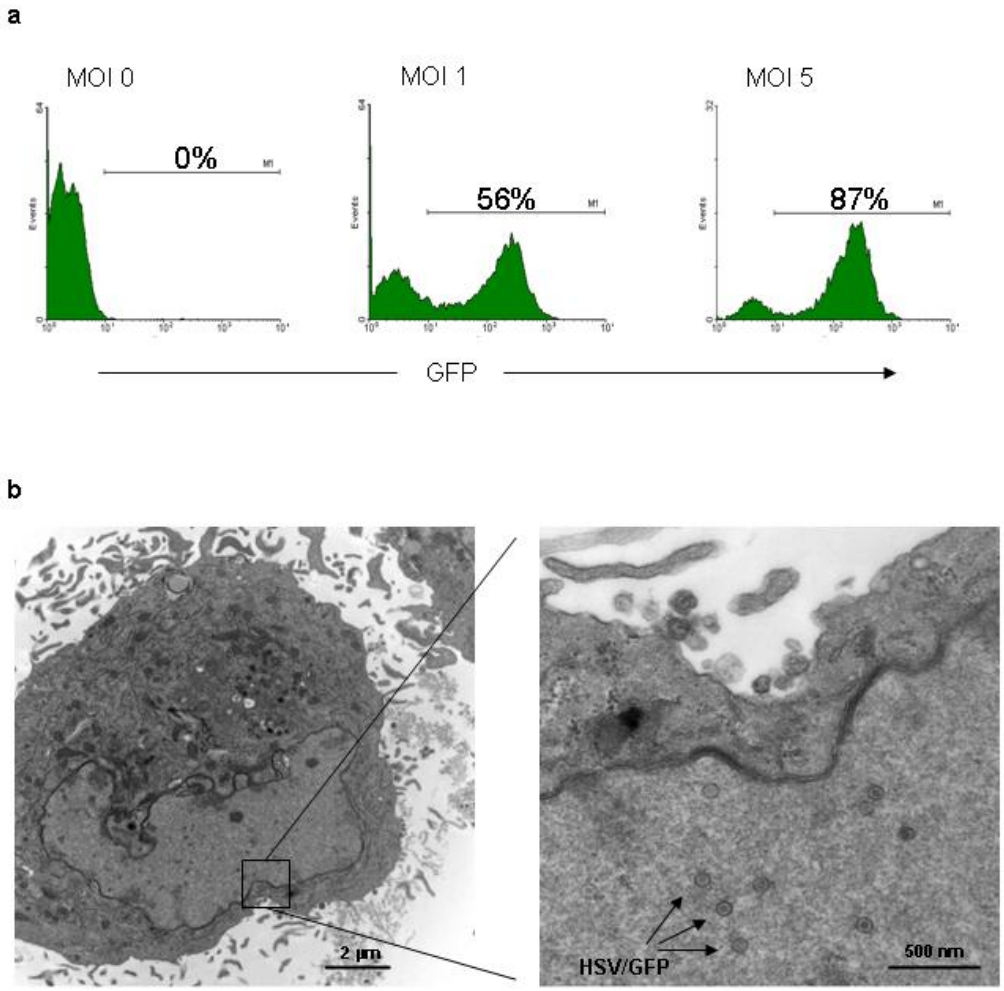


Figure 3.3: HSV-1 infection of immature DC. a) Representative experiment showing MDDC infected with HSV-1/GFP at an MOI of 0, 1 and 5 pfu/cell, and GFP expression on the R1 population (panel a) as measured by flow cytometry 18 hrs post-infection. The percentage of positive cells (as compared to FL1 signal on uninfected cells, MOI of 0) is indicated. b) Transmission EM of MDDC infected at an MOI of 1. Arrows indicating HSV/GFP.

3.2.3. Induction of MDDC maturation by HSV-1 glycoproteins

To investigate the interactions of the key HSV-1 surface glycoproteins with DC, independent of the effects of other virus components and viral infection, a model adapted from Turner et al. (Turner et al., 1998) was designed and developed, where Cos-7 fibroblasts were transfected to express the individual HSV-1 glycoproteins (gB, gD and the heterodimer gHgL). This strategy was designed to be less susceptible to LPS contamination than any strategy that was dependent on the use of purified glycoproteins, or of antibody inhibition, and allowed a much more flexible analysis of both glycoprotein combinations, and of mutant forms. Glycoprotein expression was monitored using antibodies specific for each respective glycoprotein, and examined either by flow cytometry (fig. 3.4a) or by confocal microscopy (fig. 3.4b). Optimum expression levels were observed 48 hours after transfection. Therefore Cos-7 cells that had been transfected for 48 hours were used to stimulate DC in subsequent experiments. Cos-7 cells expressing the four glycoproteins were frequently multinucleate, presumably as a result of cell-cell fusion induced by the glycoproteins (Turner et al., 1998).

A dose response analysis was used to determine the optimum Cos-7 to DC ratio. Glycoprotein expressing Cos-7 cells were cultured in an increasing number (10^2 – $5x10^4$ cells/well), co-cultured with a constant number of DC ($3x10^5$ cells/well) and then analyzed for maximum phenotypic DC response (fig. 3.4c). The data show a clear dose response with the optimum Cos-7 to DC ratio which gives the maximum DC response at 2.5 x 10^4 Cos-7 cells per 3 x 10^5 DC (ratio of approximately 1:10). This ratio was used thereafter in all subsequent experiments.

Day 7 DC were co-cultured with the TLR4 ligand, LPS, as a positive control for the induction of maturation; other controls were HSV/GFP (MOI of 5 pfu/cell giving an infection rate of around 80%); Cos-7 cells transfected previously with all four glycoproteins (gB, gD and gHgL); and mock transfected Cos-7 cells. After 18-24 hours of co-culture, all cells were collected and stained for CD13 to differentiate the MDDC from the Cos-7 cells, since CD13 (an aminopeptidase expressed exclusively on cells of myeloid origin) is a discriminatory marker that is not expressed on Cos-7 cells. $CD13^+$ MDDC cells were gated (fig 3.5) and the expression of maturation markers CD86, CD83 and HLA-DR were measured on these gated cells. Representative flow cytometry histograms from individual experiments (fig. 3.6a) and mean fluorescence levels obtained from several experiments (fig. 3.6b) are shown.

In agreement with previous studies, HSV-1 virions induced upregulation of all three surface markers, consistent with the induction of MDDC maturation, albeit the levels were statistically lower than those seen in the presence of LPS (fig. 3.6). However, Cos-7 cells transfected with all four essential HSV-1 glycoproteins also induced strong upregulation of all three surface markers (fig. 3.6 bottom panels, compared to upper panel unstimulated; fig. 3.6). Levels of CD86 detected on MDDC co-cultured with transfected Cos-7 cell were similar to those on LPS-matured MDDC and higher than that obtained by stimulation with HSV/GFP. CD83 and HLA-DR expression on MDDC varies over a broader range after exposure to transfected Cos-7 and upregulation is mostly similar to that induced by HSV virions. Mock transfected cells did not mature MDDC significantly. Thus the four HSV-1 glycoproteins are sufficient to mature MDDC independent of any other viral components.

In order to verify that DC maturation was the result of the glycoproteins and independent of the expressing cells (i.e. Cos-7 cells), CHO cells were also transfected with the HSV-1 surface glycoproteins, and co-cultured with immature DC. The CHO cells expressed similar levels of glycoproteins 48 hrs post-infection as the Cos-7 cells (data not shown). But, unlike the Cos-7 cells, these cells lack the HSV entry receptors and thus are non-permissive to HSV infections and to cell-cell fusion. Similar results of CD86 upregulation in DC were observed with all four glycoproteins compared to the Cos-7 cells (fig. 3.7).

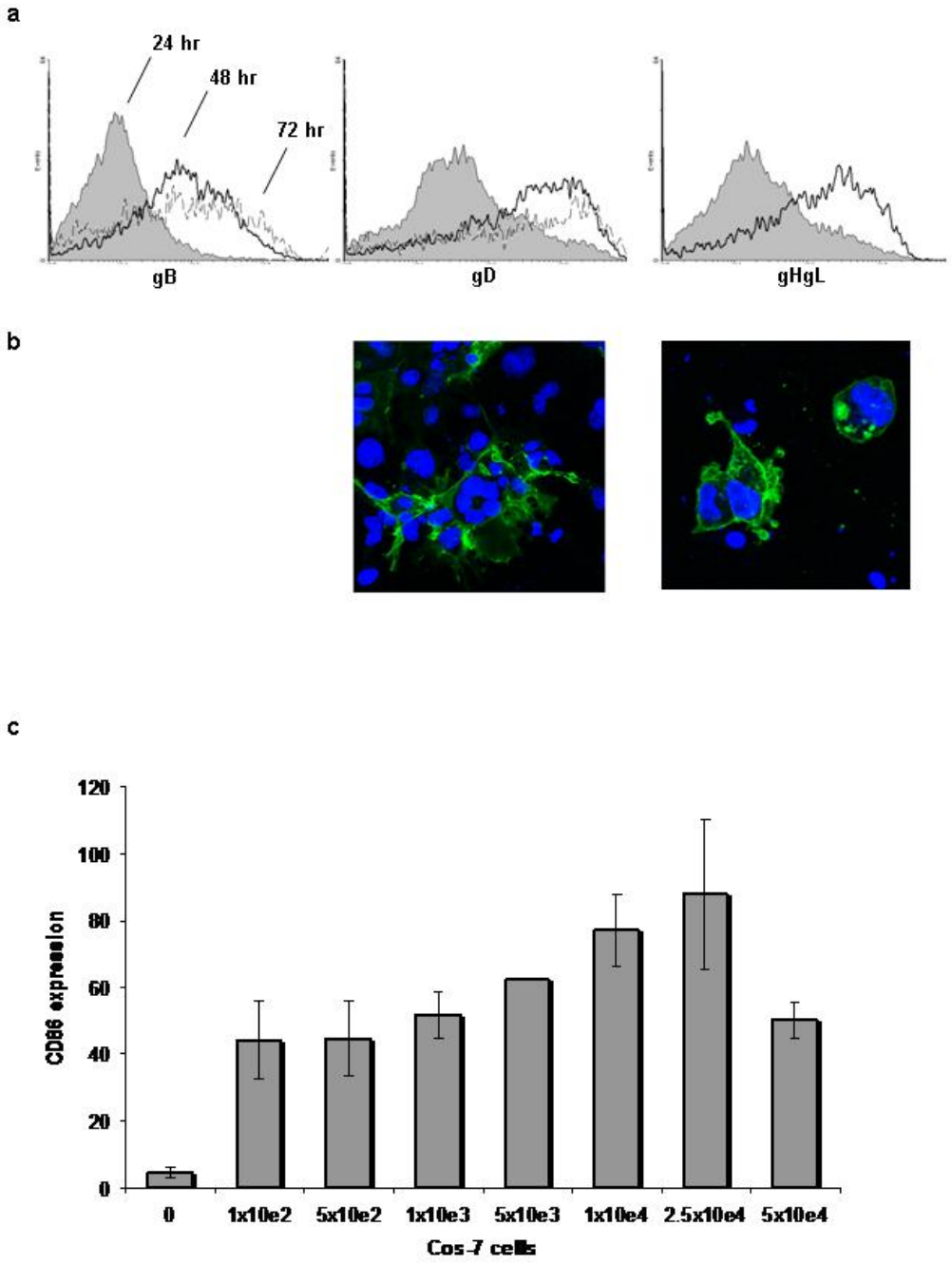


Figure 3.4: Glycoprotein expression by Cos-7 cells. Cos-7 fibroblasts were transfected transiently with vectors expressing gB, gD, gH and gL, and cultured for 24, 48 or 72h to allow expression of the glycoproteins at the cell surface. a) Expression of each glycoprotein was monitored by flow cytometry using Ab 2153 for gB, Ab LP2 for gD and Ab LP11 for gH/gL. A representative of three or more experiments is shown in each panel. b) Expression of glycoproteins following 48 hrs culture were monitored by confocal microscopy for expression of gD and gHgL. c) Glycoprotein expressing Cos-7 cells were cultured in an increasing number, co-cultured with a constant number of DC and then analyzed by flow cytometry for maximum phenotypic CD86 DC response.

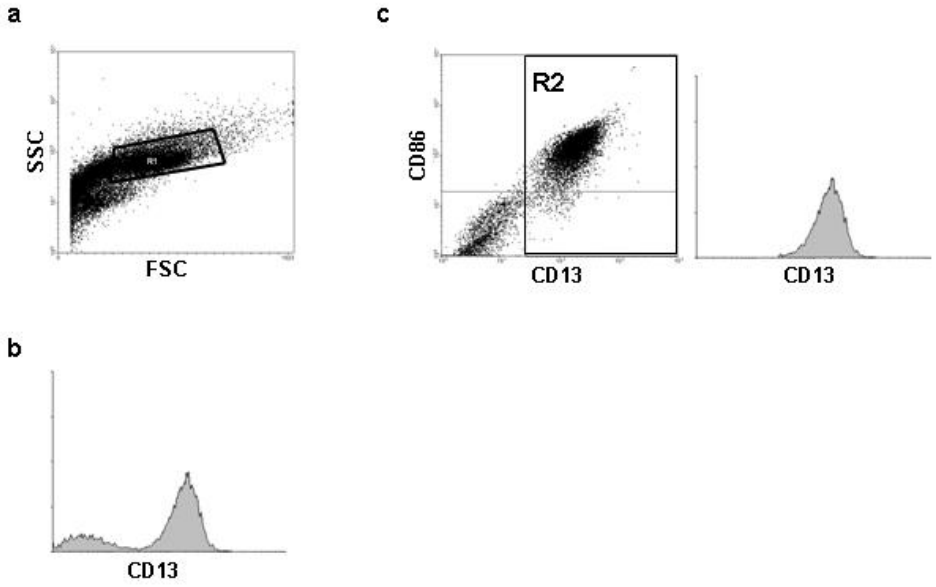


Figure 3.5: Flow cytometric analysis of MDDC/Cos-7 cells co-cultures. a) Live cells present in DC/Cos-7 co-cultures were identified by gating on the FSC/SSC region R1 and b) stained for CD13. Right peak represents the MDDC, left peak the Cos-7 cells. c) These cells were further divided into $CD13^+$ MDDC and $CD13^-$ Cos-7 cells. The levels of maturation markers (as described in the text), were analysed on MDDC alone, by gating on the $CD13^+$ region R2.

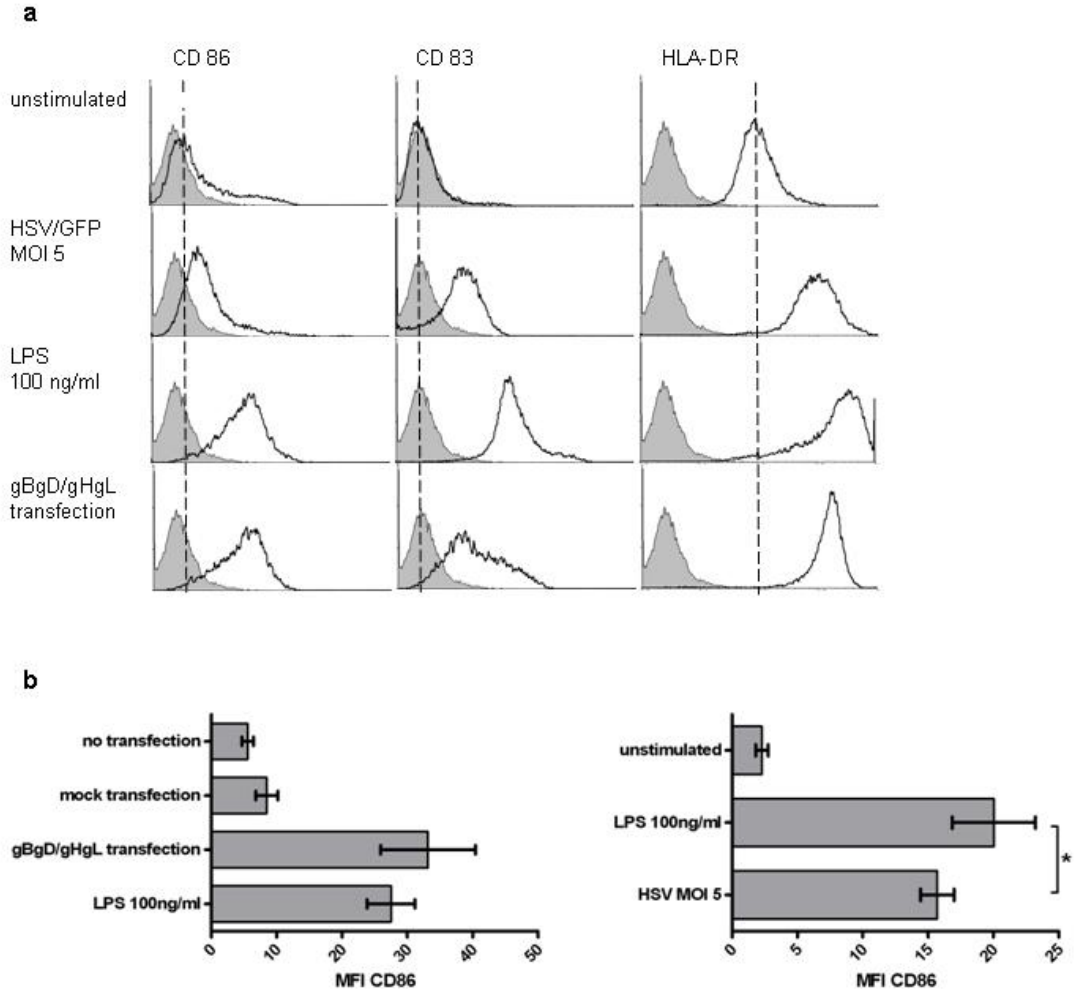


Figure 3.6: Cos-7 cells transfected with all four HSV-1 surface glycoproteins induce DC maturation. a) MDDC were cultured in medium (top row) or stimulated with either whole virus (HSV/GFP, MOI 5), LPS (100 ng/ml), or Cos-7 cells transfected with all four glycoproteins. The levels of CD83, CD86 and HLA-DR were measured by flow cytometry as in fig. 3.1c (for top three rows) or as described in fig. 3.4 for bottom row. Filled histograms show staining using an appropriate Ig control antibody. The vertical dotted line indicates the mode of the distribution in unstimulated immature MDDC. The figure shows one representative experiment from at least three independent experiments from three different donors. b) As in a, but figure shows the mean of the mean fluorescent intensity (MFI) values from three independent experiments. An additional group of MDDC co-cultured with mock transfected Cos-7 cells is included, but no significant differences were seen between mock transfected Cos-7 cells, untransfected Cos-7 cells, or MDDC alone. The experiments shown in left and right panels are shown separately since they were carried out using different batches of secondary antibody, and hence the absolute values of fluorescence cannot be compared directly. Error bars represent standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.05$.

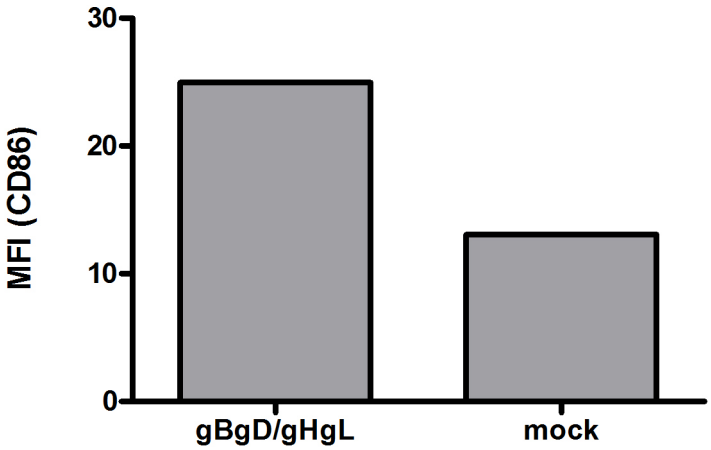


Figure 3.7: CHO cells transfected with all four HSV-1 surface glycoproteins induce DC maturation. MDDC were co-cultured with CHO cells transfected with all four HSV-1 glycoproteins, or with mock transfection, and the levels of CD86 were measured by flow cytometry. MFI values from two independent experiments from two different donors are shown.

3.2.4. All four HSV-1 surface glycoproteins are necessary for DC maturation

For viral cell entry *in vitro* (and possibly *in vivo*), all four HSV-1 glycoproteins (gB, gD and the heterodimer gHgL) are necessary as a complex. To test whether this is also true for the maturation of DC, we transfected Cos-7 cells with either one, two or three glycoproteins. Because gL on its own is not present on the cell surface and because gH requires gL for cell surface expression (Hutchinson et al., 1992; Roop et al., 1993), gH/gL was transfected as a heterodimer only. Following DC co-culture, flow cytometry showed that neither gB/gD (3.8, third row), nor gH/gL (3.8, fourth row), nor gB or gD alone (not shown) induced significant upregulation of either CD86 or CD83.

Furthermore, DC formed distinct clusters around gB/gD/gH,gL transfected Cos-7 cells resembling the features noted in HSV-1 post – infection (Pollara et al., 2004a), while no clustering was observed when mock or three or less glycoproteins were transfected (fig. 3.9). To confirm the requirement for all four glycoproteins in induction of DC maturation, glycoprotein-specific antibodies shown previously to inhibit HSV infection were added to the co-cultures. Antibodies to gHgL (LP11) and gD (AP7) inhibited DC maturation independently in the co-cultures (fig. 3.10). Antibody to gB (2153) did not block either DC activation or viral infection.

Finally, soluble gD has recently been shown to induce cell signalling (Sciortino MT et al., 2008). Therefore, the ability of recombinant gD to induce DC maturation was tested. The recombinant soluble gD used to stimulate immature day 7 DC for this assay were gD285 and gD306 (both gifts of C. Krummenacher), both derived from HSV-1 KOS and known to bind to HVEM (Rux et al., 1998). These soluble glycoproteins were pre-tested for traces of LPS contamination, using an E-Toxate Kit (Sigma) and gave a

positive result for the endotoxin (sensitivity of 2 pg/ml). However, despite this up to 25 μg/ml of recombinant gD was used to stimulate DC, and there was no maturation effect (as judged by flow cytometry) at any concentration tested (fig. 3.11). At 50 μg/ml there was a slight shift, which could be due to the LPS contamination.

3.2.5. Cell fusion is not the trigger for DC maturation

There are at least three diverse pathways used by HSV-1 in entry into different cell types that are susceptible to infection: via direct fusion with the plasma membrane, via fusion within an acidic endosome, and via fusion within a neutral endosome (Koyama AH and Uchida T, 1987; Nicola et al., 2003; Nicola et al., 2005; Milne et al., 2005). Since the complex of all four glycoproteins induces extensive cell fusion (Browne et al., 2001) it was important to determine whether the fusion event per se was the trigger for induction of MDDC maturation. One way to block fusion is to fix the glycoproteins, thus blocking the conformational changes required. Previous studies have shown that HSV-1/GFP virions fixed in 0.05% glutaraldehyde still induced CD86 and CD83 upregulation (Pollara et al., 2004a) (fig. 3.12a). The DC exposed to fixed HSV/GFP matured further in response to LPS, confirming their viability. Cos-7 cells transfected with all four glycoproteins, and then fixed in 0.05% glutaraldehyde, also induced DC maturation as efficiently as unfixed cells (fig. 3.12b). Thus fixation does not block the ability of the glycoproteins to induce DC maturation.

Various reports have suggested gHgL is one of the components required for viral fusion during HSV-1 entry , and the fusion domain within gH has been studied extensively (Forrester A et al., 1992; Gianni et al., 2005b; Gianni et al., 2005a). Therefore we confirmed that fusion was not necessary for the induction of DC maturation by HSV glycoproteins, by using gH mutants (see Materials and Methods) that lacked fusogenic

activity (Gianni et al., 2005b; Gianni et al., 2005a; Gianni et al., 2006). We transfected these mutants together with wild-type gB, gD and gL into Cos-7 cells. In the Cos-7 cells expressing all four wild-type glycoproteins, more than 90% of cells expressing gD were observed to be multinucleated, as a result of glycoprotein-induced Cos-7 cell fusion (fig. 3.13a, second panel). In contrast, Cos-7 cells transfected with gB, gD, gL and any of the mutant gH glycoproteins showed no evidence of multinucleate cells (fig. 3.13a, panel 3-5) confirming that these mutants were unable to induce cell fusion. However, Cos-7 cells transfected with the mutated gH were similar to wild type gH transfection in their ability to induce DC maturation (fig. 3.13b, CD83 data not shown). In agreement with the data obtained using fixed transfected Cos-7 cells, this data suggests that membrane fusion is not required for HSV-1 glycoprotein-induced MDDC activation.

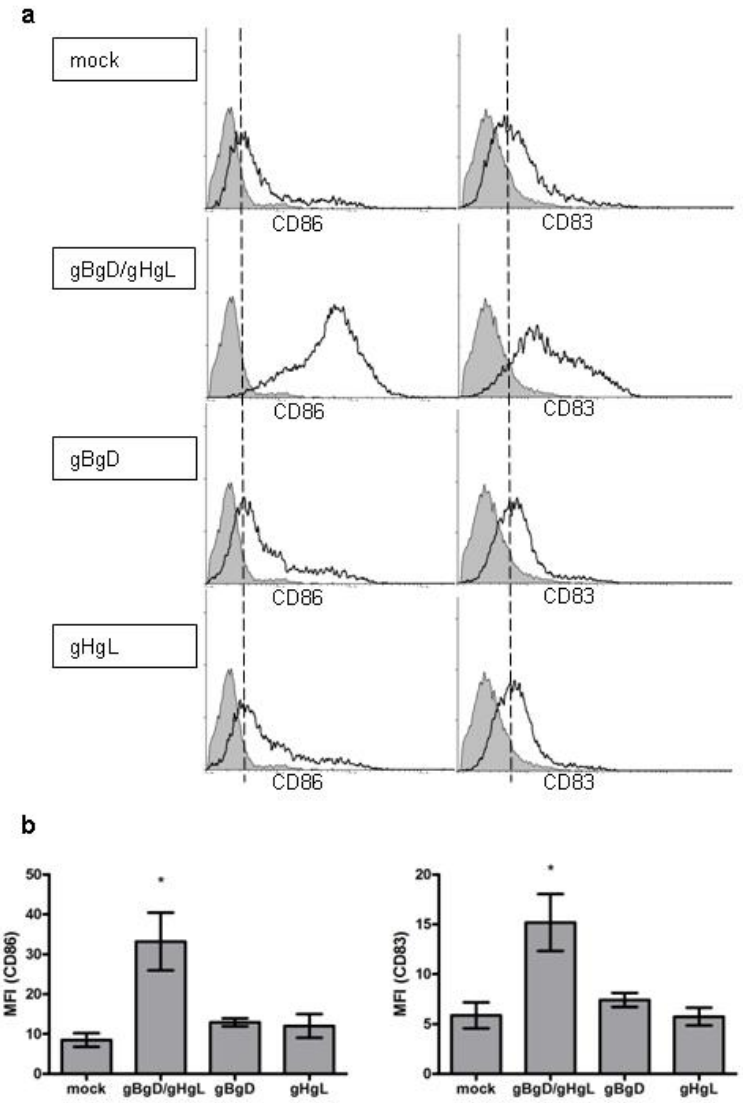


Figure 3.8: All four HSV-1 surface glycoproteins are necessary for phenotypic changes in DC. a) MDDC were co-cultured for twenty four hours with mock transfected Cos-7 fibroblasts, or Cos-7 cells transfected with either gBgDgHgL, gBgD only or gHgL only. Expression of CD86 and CD83 on $CD13^+$ MDDC was measured by flow cytometry as described in Legend to fig. 3.4 (white histogram). The grey histogram represents IgG control staining. The dotted line indicates the mode of the distribution in unstimulated immature MDDC. The figure shows one representative experiment from at least three independent experiments. b) As in a, but figure shows the MFI values from three independent experiments. Error bars represent the standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.01$.

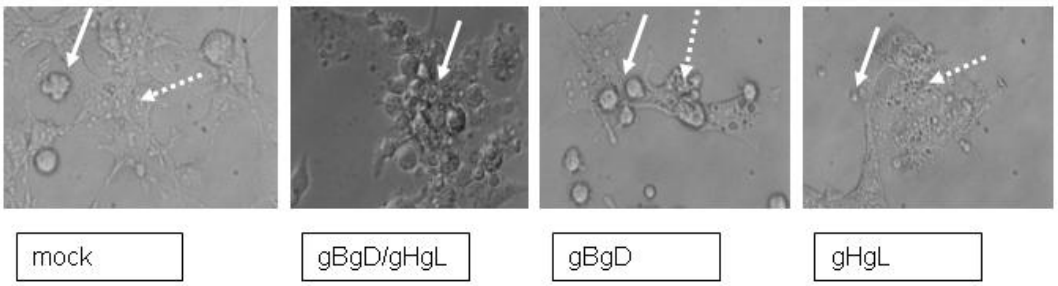


Figure 3.9: All four HSV-1 surface glycoproteins are necessary for morphological changes in DC. Images of the cells in culture after twenty four hours were obtained by inverted phase contrast microscopy, showing clustering of DC on Cos-7 cells expressing all four glycoproteins. Filled arrows indicate MDDC, dashed arrows indicate Cos-7 cells.

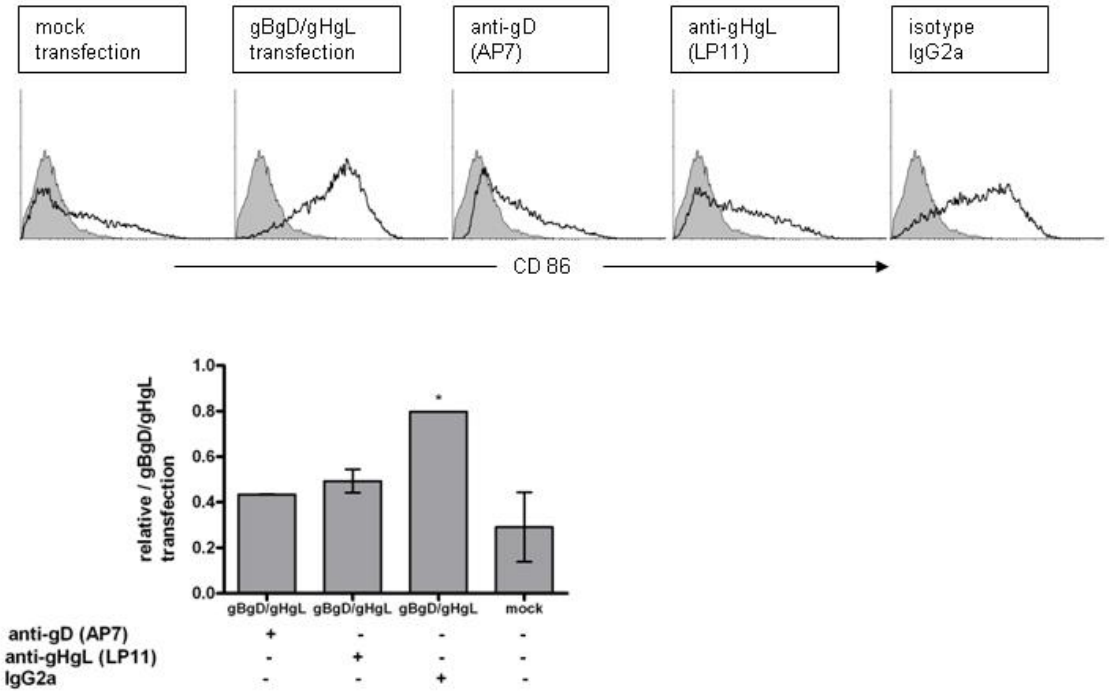


Figure 3.10: Anti-glycoprotein antibodies inhibit MDDC maturation. MDDC were co-cultured for twenty four hours with Cos-7 cells transfected with gBgDgHgL, and anti-gD (AP7) or anti-gHgL (LP11) specific antibodies or IgG2a control antibodies. The antibodies were added to the Cos-7 cells 1 hour prior to the addition of MDDC. CD86 was measured by flow cytometry (white histograms). Grey histogram represents IgG staining control. Results are representative of three or more independent experiments. Lower histogram show MFI values relative to MFI of MDDC co-cultured with all four glycoproteins from three independent experiments. Error bars represent standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.05$.

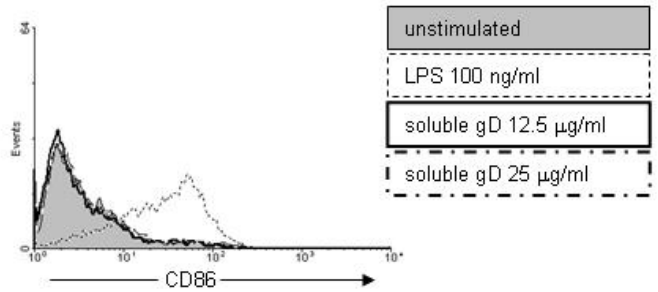


Figure 3.11: Soluble gD does not induce MDDC maturation. MDDC were stimulated with increasing concentrations of soluble gD (12.5 g/ml and 25 µg/ml) or LPS (100 ng/, dotted histogram). Expression of CD86 was measured by flow cytometry. Grey histogram represents immature unstimulated MDDC.

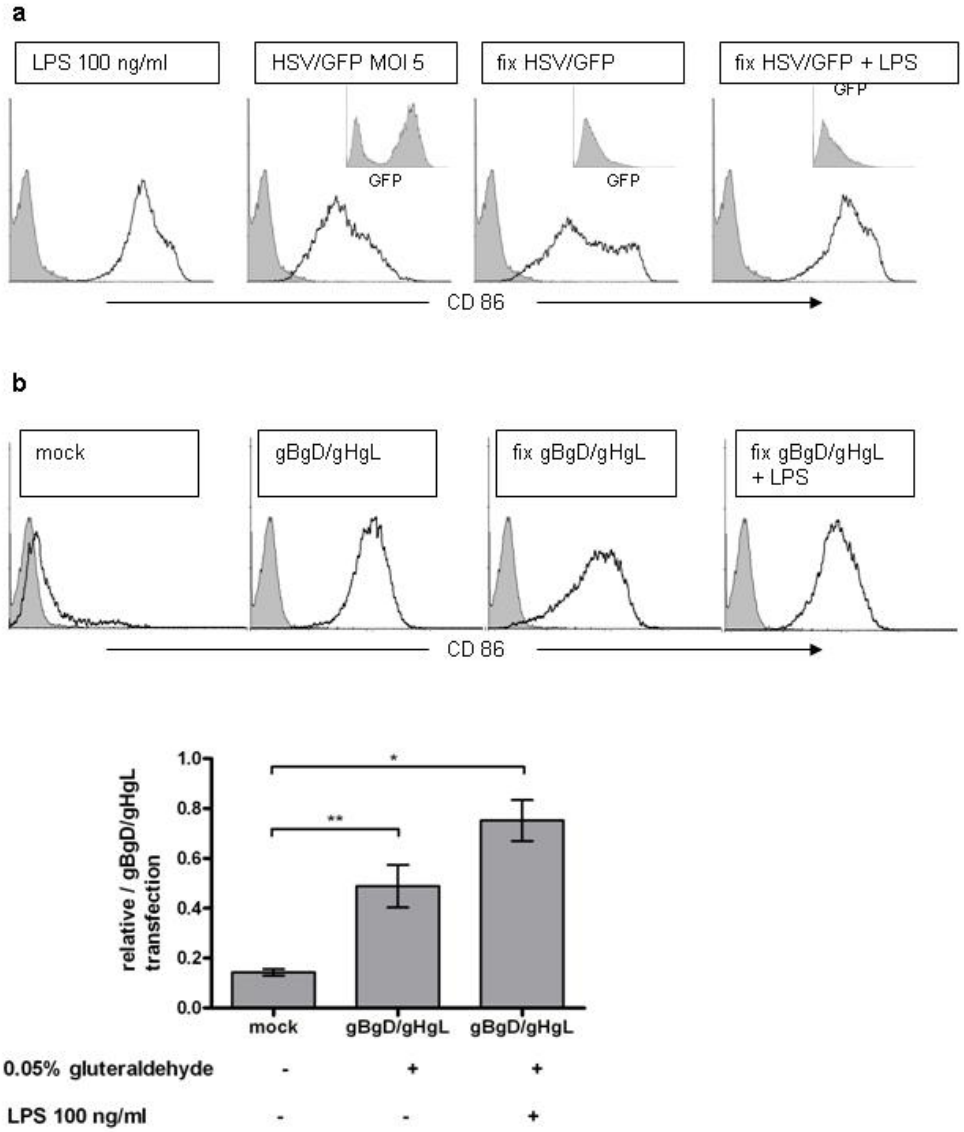


Figure 3.12: Glutaraldehyde fixation inactivates HSV-1, but does not block glycoprotein mediated MDDC maturation. a) MDDC were cultured with LPS (100 ng/ml), viable HSV/GFP (MOI 5), 0.05% glutaraldehyde fixed HSV/GFP, or both LPS and fixed HSV/GFP. Expression of CD86 was analysed after 18 hours (white histograms). The inserted histograms show GFP expression to confirm that the HSV/GFP has been inactivated by the concentration of glutaraldehyde used in these experiments. b) Immature DC were co-cultured with mock transfected Cos-7 cells, viable Cos-7 cells transfected with all four glycoproteins , Cos-7 cells transfected with all four glycoproteins and then fixed with 0.05% glutaraldehyde, and fixed transfected Cos-7 cells and LPS (100 ng/ml). Expression of CD86 was analysed after 18 hours co-culture (white). Grey histogram represents isotype control for staining. Figures show one representative of three experiments. Bottom figure shows the MFI values relative to MFI of MDDC co-cultured with all four glycoproteins from three independent experiments. Error bars represent standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.01$, ** $P < 0.05$.

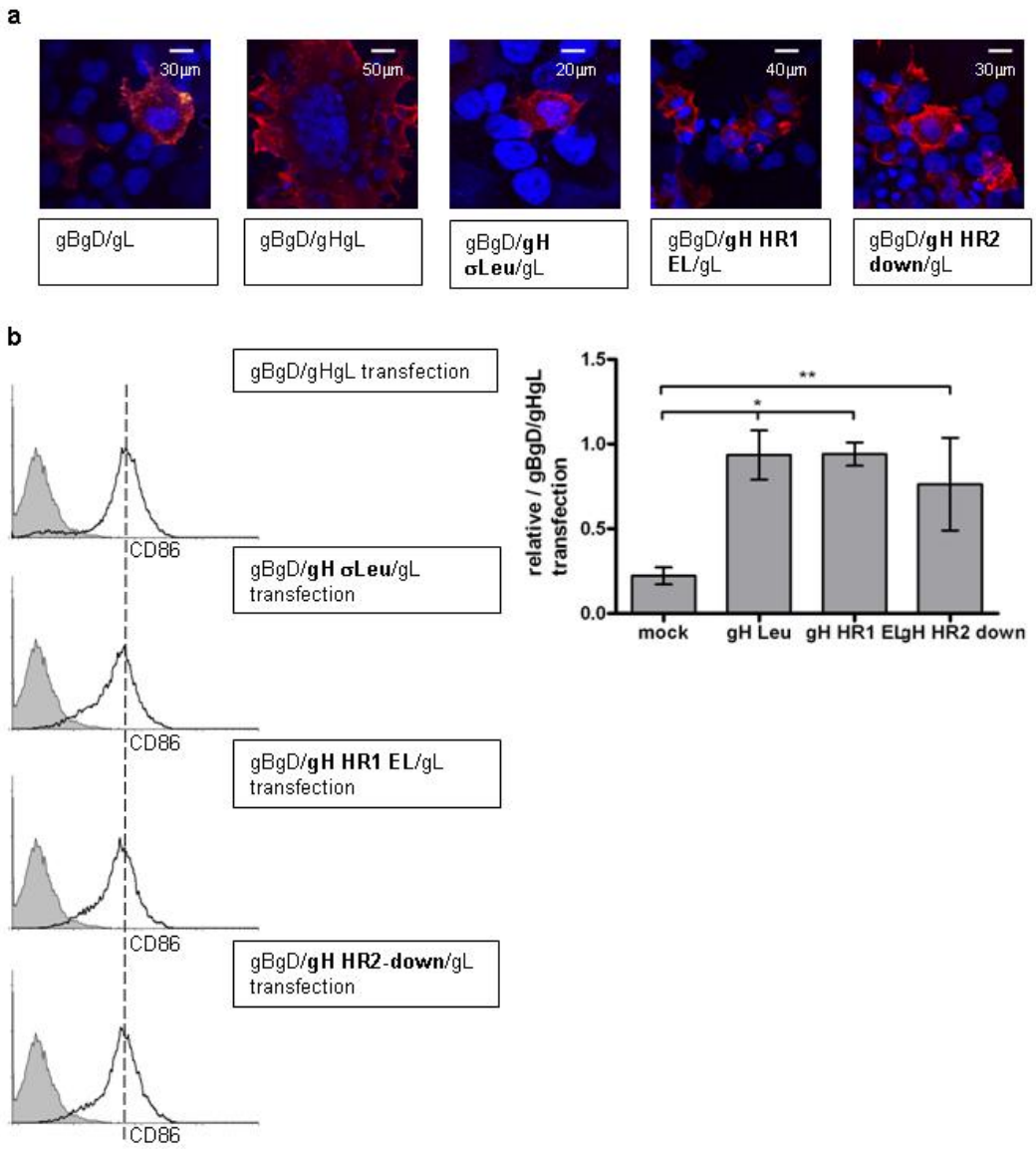


Figure 3.13: gH mutants lacking fusogenic activity do not induce DC maturation. Control transfected Cos-7 cells (gBgD/gL) , or Cos-7 cells transfected with gBgDgHgL, or wild type gB, gD, gL together with mutants of gH, were cultured for 48 hours and stained for gD (to identify transfected cells) and DAPI (to identify nuclei). Presence of multinucleated cells was detected by confocal microscopy. The figures show a high magnification view of a single field; examination of multiple high power fields showed similar findings. b) Expression of CD86 on $CD13^+$ DC was measured by flow cytometry in MDDC co-cultured with Cos-7 cells transfected with either all four wild type glycoproteins gBgDgHgL, or with wild type gBgDgL and mutant gH (white histogram). The grey histogram represents immature MDDC. The vertical dotted lines represent the levels of these markers in mature DC. The figure shows one representative experiment from at least three independent experiments. Bar chart shows the MFI values relative to MFI of MDDC co-cultured with all four glycoproteins from three independent experiments. Error bars represent standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.01$, ** $P < 0.1$.

3.2.6. The DC response to HSV-1 glycoproteins is independent of the known HSV-1 receptors

HSV-1 interaction with susceptible cells is thought to occur via the interaction of the surface glycoproteins with one of several potential receptors. Currently HVEM and nectin-1 are the two receptors known to be expressed on DC that may interact with gD. More recently an integrin co-receptor, PILRalpha, has also been implicated, associating with gB during entry. These glycoprotein/receptor interactions are known to be necessary for entry; it is also possible that the interaction transduces activation pathways necessary for maturation. To test this latter possibility three gD mutants were used, one of which cannot bind HVEM [gD(D30A)], one of which cannot bind to nectin-1 [gD(A3C,Y38C)] and one of which binds neither receptor [gD(V37C-A302C)] (Krummenacher et al., 2005; Connolly et al., 2003; Connolly et al., 2005). Remarkably, all three mutants induced MDDC maturation when expressed together with gB and gH/gL, although levels of CD86 were consistently lower than those observed with wild type gD (fig. 3.14).

There are at least two known TLR pathways associated with HSV-1 infections although the ligand of the receptor on the virion has not been identified. One pathway involves detection via TLR-9 and is used by pDC to recognize HSV-1 and HSV-2 (Lund et al., 2003; Krug et al., 2004); the other involves TLR-2 (Sato et al., 2006). In order to test if the glycoprotein-induced DC activation described thus far is mediated via these two pathways a HEK-293 cell line expressing the specific individual TLR was used. Upon triggering via the TLR these cells secrete IL-8 which can be detected by ELISA. As shown in figure 3.15, TLR-2 ligand Pam3Cys and TLR-9 ligand CpG were both powerful activators of IL-8 secretion in this system. In contrast, Cos-7 cells expressing

all four glycoproteins, similar to mock transfection failed to stimulate any significant IL-8 secretion.

To confirm the finding that the glycoprotein induced maturation was independent of TLR-2 and TLR-9, Cos-7 cells expressing the four entry glycoproteins were co-cultured with monocyte-derived Langerhans cells (MDLC) which have been shown previously to express no or very low levels of TLR-2 (Takeuchi et al., 2003; van der Aar et al., 2007) and plasmacytoid DC (pDC) in which HSV-1 has been shown to trigger TLR-9 (Lund et al., 2003). These results will be shown and discussed in chapter 5.

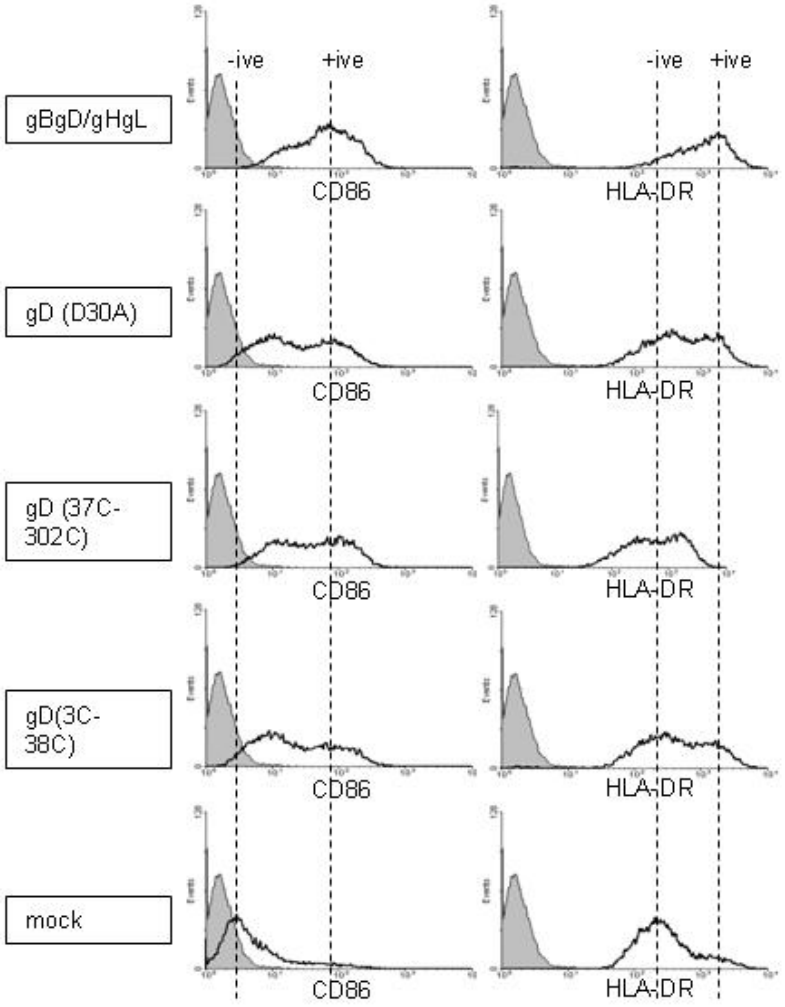


Figure 3.14: DC maturation response to viral glycoproteins does not require binding to HVEM and/or nectin-1. MDDC were co-cultured for twenty fours hours with Cos-7 cells transfected with either all four wild type glycoproteins gBgDgHgL, or with wild type gBgHgL and mutant gD as shown. Expression of CD86 and HLA-DR on $CD13^+$ DC was measured by flow cytometry (white histogram). The grey histogram represents IgG control staining. The vertical dotted lines represent the mode of the distribution for immature (left line) and LPS matured (right line) MDDC. The figure shows one representative experiment from at least three independent experiments from three different donors.

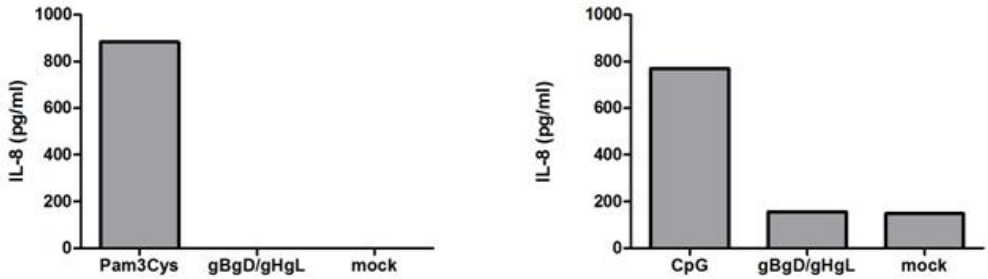


Figure 3.15: DC response to HSV-1 glycoproteins is independent of TLR-2 and TLR-9. HEK-293 cells stably expressing TLR-2 or TLR-9 were stimulated with TLR ligand (Pam3Cys for TLR-2; CpG for TLR-9) or were co-cultured with Cos-7 cells expressing either all four HSV-1 entry glycoproteins (gBgD/gHgL) or mock transfection. After an overnight co-culture, cells were analyzed for secreted IL-8 on an ELISA plate reader at 450 nm. Histograms represent the mean value of triplicate readings. The figure is one representative experiment from two independent experiments.

3.3. Discussion

It has been shown previously that both UV inactivated virus and formaldehyde fixed virus (which are unable to express any de novo viral gene products) are still able to induce DC to mature, and this is reflected in resultant increase in T cells activation (Pollara et al., 2004a). This suggested that activation is mediated by components of the viral particle itself. The viral particle, however, contains many components which could in principal mediate DC activation. Therefore the first hypothesis tested in this study was whether or not the four entry glycoproteins, gB, gD, gH and gL, that are known to be involved in entry and membrane fusion, function as inducers of maturation in DC.

In order to establish this role of HSV glycoproteins in the absence of other viral components we have adapted a model developed initially for the study of glycoprotein-mediated attachment and fusion (Browne et al., 2001). In this model, DC are stimulated by Cos-7 cells expressing HSV-1 glycoproteins on their surface as a surrogate for exposure to intact viral particles. This strategy has proved robust, much less susceptible to LPS contamination than is seen when using purified recombinant glycoproteins, and allows a very flexible structure/function analysis of the role of individual glycoproteins and their combinations. This model system was then used to show clearly that the four entry glycoproteins were able to induce DC phenotypic maturation without requirement for any other viral component. This recognition pathway may be important *in vivo*, since glycoproteins are expressed at high levels on infected keratinocytes within HSV lesions (Cunningham AL et al., 1985). In a preliminary study looking at a keratinocyte cell line, HaCaT cells demonstrated a high susceptibility to HSV-1 infection and surface expression of the viral glycoproteins within a 24 hour infection (Appendix A). Interaction between DC and infected keratinocytes may provide one important signal for the initiation of adaptive immunity.

In principle the complex of four glycoproteins on Cos-7 cells is sufficient to allow fusion between Cos cells and DC (Reske A et al., 2007). Fusion in itself could provide a trigger for DC maturation, for example by introducing Cos cell DNA into the DC cytoplasm (Takaoka et al., 2007) rather than via the interaction between individual glycoproteins and their receptors. Two sets of experiments were carried out to test this hypothesis. In the first set, the transfected Cos-7 cells were fixed briefly with gluteraldehyde, which completely blocks fusion, before co-culture with the DC. In the second, a set of gH mutants with non-functional fusion domains was used (Gianni et al., 2005b; Gianni et al., 2005a; Gianni et al., 2006). Both sets of experiments demonstrated unequivocally that DC maturation induced by the glycoproteins is independent of membrane fusion.

DC recognition of HSV-1 can therefore occur in the absence of other viral components, and in the absence of fusion. These results suggested strongly that a receptor (or receptors) is present on the DC surface, which is able to interact with the glycoproteins, and transmit an activating signal to the DC. Several receptors of the individual HSV-1 glycoproteins have been described already (Spear, 2004; Satoh et al., 2008), with different distribution on different cell types. Thus we tested whether any of the glycoproteins individually could induce DC maturation via interaction with their respective receptors. Unexpectedly, DC maturation was found to require the co-operative interaction of all four glycoproteins. The requirement for co-operative interactions was demonstrated both by transfection of each glycoprotein alone, or in various combinations, and also by the use of blocking antibodies to gD and gH. Both approaches confirmed the synergism between gB, gD and gH in triggering DC maturation.

Of the known gD receptors, HVEM, a TNF receptor family member, signals via NF-κB in response to its natural ligand LIGHT (Marsters et al., 1997; Tamada et al., 2000; Hikichi et al., 2001). Signalling via this receptor might therefore be an essential, though clearly not a sufficient event for the DC maturation that was seen in our Cos-7 model. In order to test this hypothesis a set of three gD mutants were used, which are unable to bind to HVEM, nectin-1 or either receptor respectively. All three mutants induced DC activation when used in conjunction with the other glycoproteins, albeit to a slightly lesser extent than the wild type protein. Thus the direct interaction between gD and HVEM or nectin-1 on the DC surface does not appear to be essential or sufficient for initiating DC maturation. It should be noted, however, that in receptor studies, one cannot rule out the possibility that in the absence of one receptor, gD binds to the other receptor, or to a totally different receptorthat has not as yet been identified.

Finaly, the role of TLR-2 and TLR-9 in glycoprotein recognition was also excluded. These receptors have a well established role in innate responses to HSV-1 (Lund et al., 2003; Krug et al., 2004; Sato et al., 2006); however in this study their direct interaction with the four viral glycoproteins tested is not essential.

3.4. Conclusion

In this chapter a method developed for the study of HSV-1 entry was used to unravel and understand the interactions between viral surface glycoproteins and MDDC. The results demonstrate, using a variety of different experimental approaches that in HSV-1 maturation of DC all four known entry glycoproteins are necessary and sufficient and that this maturation is independent of the known HSV-1 receptors. This suggests that the initial interaction between the glycoproteins and DC is the trigger for maturation, is not dependent upon any other viral components.

This model and the results described will be used in the following chapters to further understand the interactions between the viral glycoproteins and DC.

CHAPTER 4 – THE BIOLOGICAL CONSEQUENCE OF MDDC INTERACTION WITH THE HSV-1 GLYCOPROTEINS

4.1. Introduction

DC are the most potent antigen presenting cells, highly efficient at migrating to the lymph nodes to initiate primary T-cell mediated immune responses and also contributing to the release of pro-inflammatory cytokines and chemokines necessary for control of initial infection before adaptive immunity is operative.

The role of these cytokines and chemokines as key effector molecules in all innate immune responses (whether triggered by viruses, or by other forms of injury) has been studied intensively in recent years, based upon new ideas about the significance of this phase of the reaction. With respect to HSV-1, the release of type I interferons (IFN) is an important component of the innate immune response to HSV-1 (Leib et al., 1999; Noisakran et al., 2000) and genetic defects in type I IFN production have been shown to increase susceptibility to HSV-1 encephalitis (Noisakran et al., 2000). Several pathways, both TLR-dependent and TLR-independent pathways have been described which lead to the production of type I IFN in innate immune cells as a consequence of viral infections. In these pathways, the nuclear factor κB (NF-κB) and the interferon-regulatory factors (IRFs) have been shown to be important in regulation of type I IFN at the molecular level.

4.1.1. NF-κB

NF-κB is a key regulatory component in the control of both innate and adaptive immunity (Li and Verma, 2002). There are several subunits of NF-κB and several pathways leading to activation. In the classical (canonical) pathway, RelA/p65 is bound initially to IκB in an inactive form. Following activation, IκB proteins are phosphorylated by IκB kinases (IKK), ubiquitinated and eventually degraded allowing

the now unbound NF-κB protein to translocate to the nucleus to be subsequently phosphorylated in order to regulate the transcription of a variety of genes. Two pathways have been described for the activation of IκB degradation: a MyD88-dependent pathway that promotes an immediate NF-κB activation and induction of pro-inflammatory cytokines; and a MyD88-independent pathway which mediates a late activation of NF-κB and the induction of IFN genes and co-stimulatory molecules (Kawai et al., 1999; Kaisho and Akira, 2001; Kawai et al., 2001; Kaisho et al., 2001; Moynagh, 2005). Both TLR3 and TLR4 have been shown to use the MyD88-independent NF-κB activation pathway in the induction of type I IFN, specifically IFNβ, and this involves phosphorylation of IRF3 (see below), which then co-operates with other transcription factors (Kawai et al., 2001; Doyle S et al., 2007). TLR7/8 and TLR9, which use the MyD88-dependent pathway, induce type I IFN via IRF7.

4.1.2. IRF

The mammalian IRF family consists of nine members, IRF1-9 (Taniguchi T et al., 2001; Lohoff M and Mark TW, 2005). Of these, IRF3 and IRF7 have received most attention as the main regulatory factors for viral induced type I IFN (Yoneyama M et al., 1998; Sato et al., 1998b; Sato et al., 1998a; Marie I et al., 1998). Both proteins are found in the cytosol. IRF3 is expressed constitutively, whereas IRF7 is found in small amounts and is induced in an autocrine fashion by type I IFN-mediated signalling. Following viral infection, these proteins are phosphorylated and translocate to the nucleus to interact with the type I IFN genes. IRF3 is a potent activator of IFNB genes, but not IFNA genes, except for IFNA4. IRF7 activates both IFNA and IFNB genes efficeintly (Sato et al., 1998a; Marie I et al., 1998; Sato et al., 2000). Studies on IRF7 knock-out mice (Honda et al., 2005) suggested a positive-loop model in which the small

amounts of IRF7 in the cytosol are responsible, as homodimers or heterodimer with IRF3, for the initial induction of IFNβ, whose binding to the IFN receptor (IFNR) brings about an upregulation of more cellular IRF7 which is responsible for the late phase and continuous production of the type I IFN (fig. 4.1).

Both the TLR-dependent (TLR3, TLR4, TLR7/8 and TLR9) and the TLR-independent (RIG-I and MDA-5) forms have been identified as inducers of type I IFN genes via the IRF3 and IRF7 pathways in response to viral infections (Honda et al., 2006; Honda and Taniguchi, 2006).

In chapter 3, the four viral entry glycoproteins were shown to be essential and sufficient to induce phenotypic maturation of MDDC. This chapter will study the biological consequences of the interaction between the four viral entry glycoproteins and MDDC, looking at the ability to activate T cells, cytokine production and the distinct signaling pathways that follow this interaction.

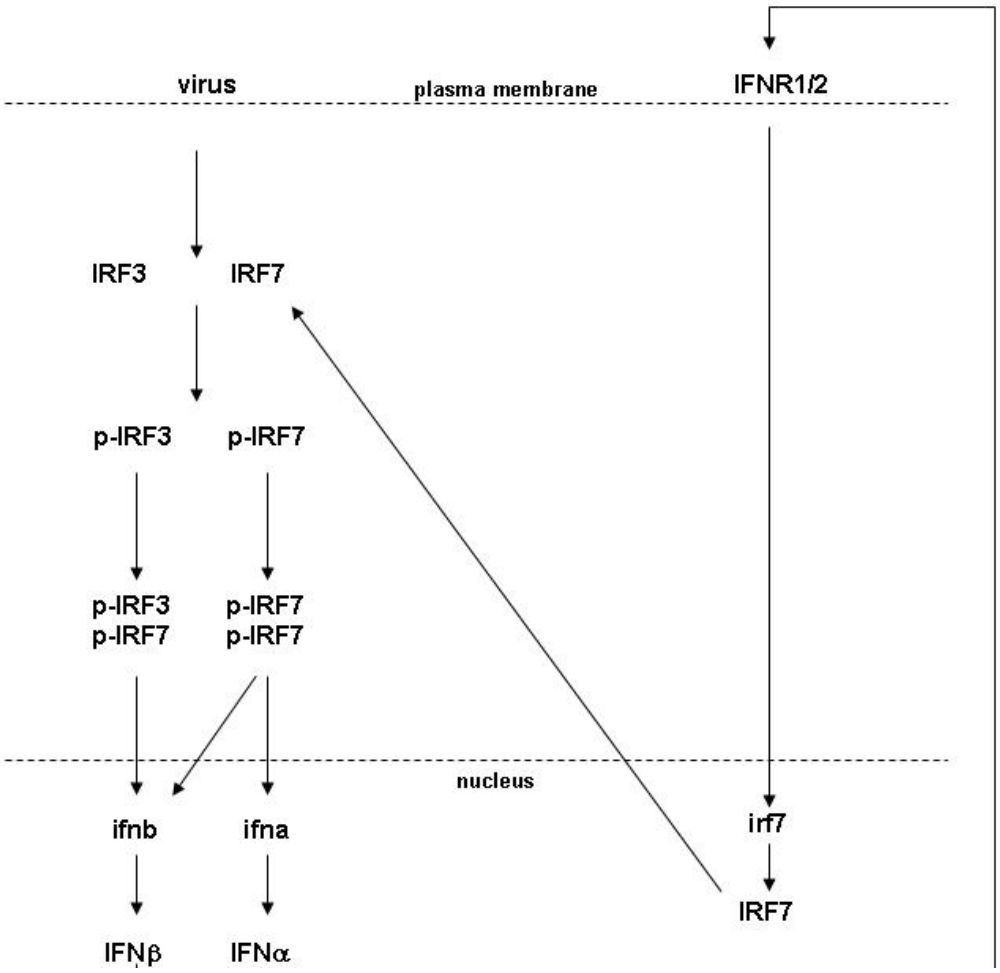


Figure 4.1: Schematic diagram of IRF3/7 positive-loop regulation of type I IFN. In the early phase of infection, both IRF3 and IRF7 are phosphorylated resulting in either homodimerization of IRF7 or heterodimerization of IRF3 and IRF7. These dimers then translocate to the nucleus where they induce the production of small amounts of IFNβ and IFNα from the ifnb and ifna genes, respectively. Secreted type I IFN can then bind with the IFNR in an autocrine or paracrine manner to induce the transcription of the irf7 gene. The IRF7 produced returns to the cytosol to induce the production of large amounts of type I IFN.

4.2. Results

4.2.1. MDDC exposed to the four viral entry glycoproteins do not induce enhanced T cell proliferation

Having shown that the four HSV-1 glycoproteins are able to up-regulate DC maturation markers, the comparative ability of these mature MDDC to induce allogeneic T cell proliferation was measured in a T cell proliferation assay. Similar to previous studies (Pollara et al., 2003), HSV-1 virions impaired the DC ability to induce T cell proliferation. In contrast to LPS stimulated MDDC which induced a significant increase in T cell proliferation compared to untreated controls, MDDC matured in the presence of Cos-7 cells expressing the HSV-1 glycoproteins were similar to unstimulated and mock transfected MDDC, with no increased T cell stimulating capacity (fig. 4.2a).

Similar results were seen when the MDDC were stained for CCR7, a G protein-coupled chemokine receptor expressed on naïve T and B cells and mature/activated DC, and shown to control the migration of mature DC to lymph nodes (Dieu MC et al., 1998; Sallusto F et al., 1998). Unstimulated MDDC, HSV-1 infected MDDC and MDDC co-cultured with transfected Cos-7 cells did not show an upregulation of CCR7 expression. The positive control, CD40L induced upregulation of CCR7 on MDDC as expected (fig. 4.2b).

Next the cytokines secreted by the MDDC responder T cells were measured. Supernatants were collected from the mixed MDDC-T cell allogeneic cultures 24 hours and 6 days post co-culture, and cytokine levels [IFN-γ (T_H1), IL-13 (T_H2) and IL-17A (Treg)] were measured by ELISA (fig. 4.3). At 24 hours, only MDDC that had been stimulated with LPS induced T cell IFN-γ synthesis. There was no IL-13 and no IL-17A release after 24 hours in any of the cultures. After 6 days in culture however, MDDC

stimulated with both LPS and poly I:C induced T cell release of IFN-γ. This was also seen with MDDC co-cultured with Cos-7 cells expressing the four viral entry glycoproteins (fig. 4.3a). T cells co-cultured with MDDC matured by LPS, poly I:C, HSV-1 and the viral glycoproteins also produced low amounts of IL-13 (fig. 4.3b). IL-17A cytokine was not detectable at either of the time points.

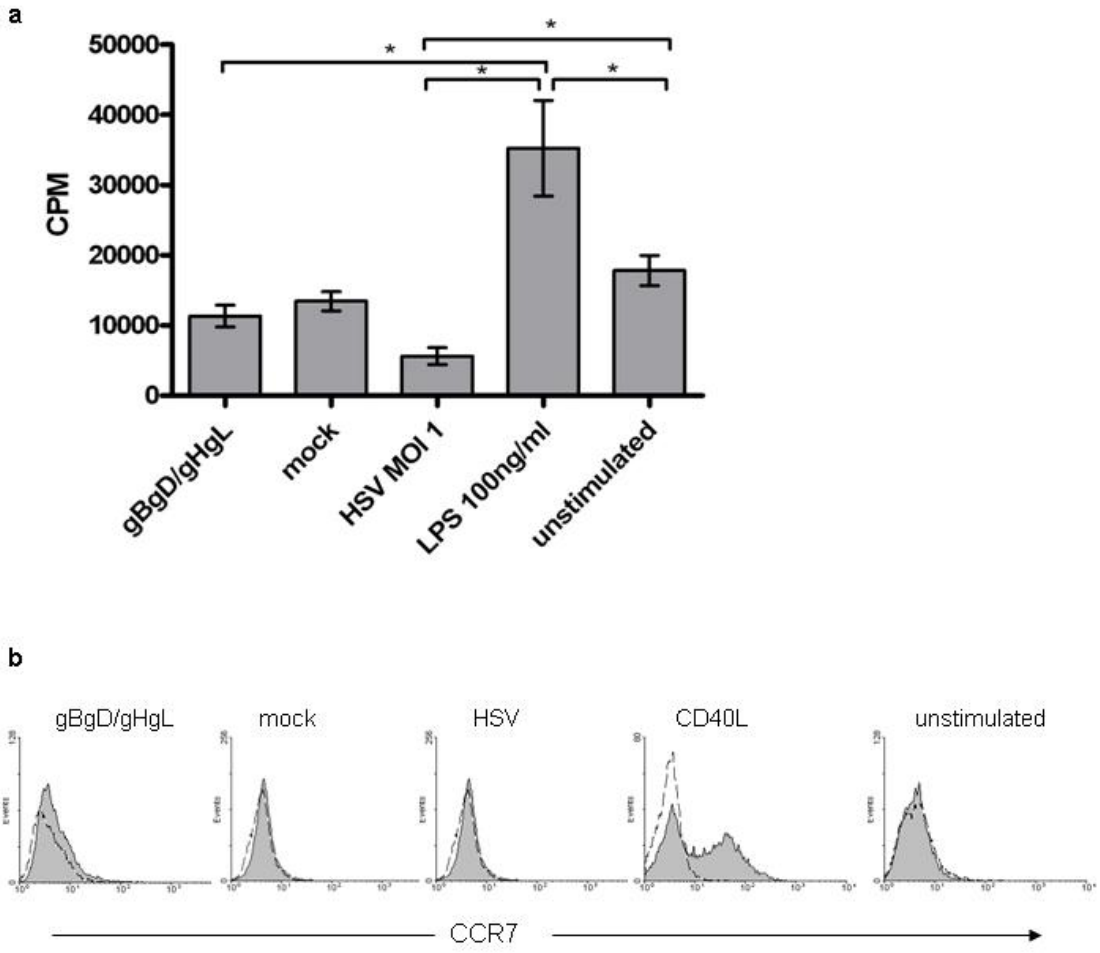


Figure 4.2: DC matured by Cos-7 cells transfected with all four glycoproteins do not induce allogeneic T cell activation. a) MDDC co-cultured with Cos-7 cells expressing the four glycoproteins, mock transfected, or infected with HSV (MOI 1) were used to stimulate allogeneic T cell proliferation. MDDC activated with LPS (100 ng/ml) and MDDC without any activation stimulus were used as controls. Results are expressed as mean counts per minute (cpm) of triplicate wells of T cells. Error bars represent standard error of the mean. Statistical analysis was performed using the Student's *t*-test. * $P < 0.05$. A representative experiment from three independent experiments from three different donors is shown. b) Immature MDDC were co-cultured with Cos-7 cells expressing four glycoproteins, mock transfection or infected with HSV, and stimulated with CD40L or unstimulated. CCR7 expression was measured by flow-cytometry 18 hrs post-stimulation. Dotted histograms represent the isotype control. A representative experiment from three independent experiments from three different donors shown.

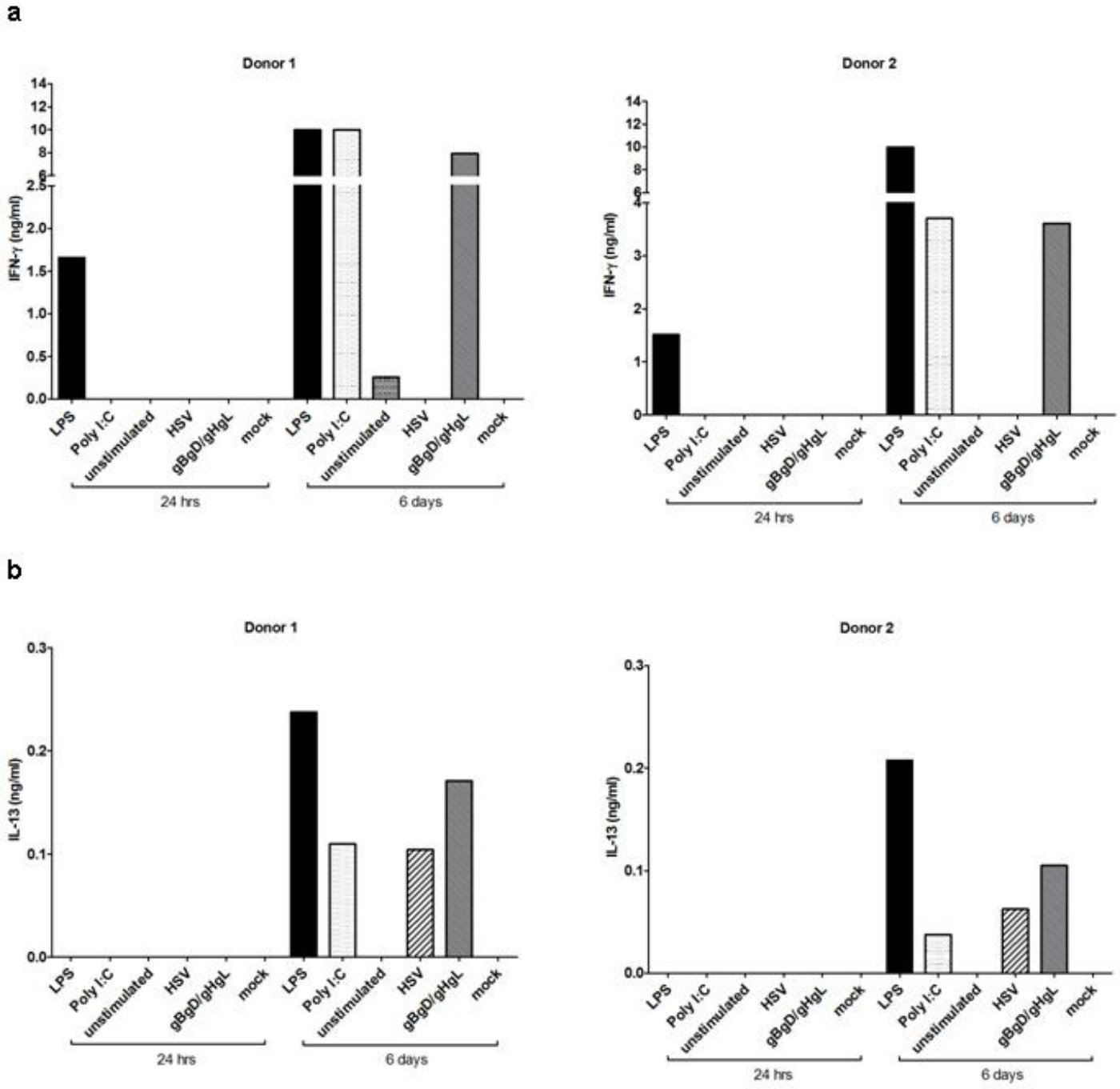


Figure 4.3: ELISA for T cell T_H1 and T_H2 cytokines. Supernatants from allogeneic T cells co-cultured with MDDC activated by LPS, poly I:C, HSV and Cos-7 cells transfected with all four glycoproteins were collected 24 hours and 6 days post-co-culture. T cell secreted a) IFN-γ and b) IL-13 were determined by ELISA. All ELISA experiments were performed in duplicates. The figures show two representative experiments from two different donors from three independent experiments.

4.2.2. HSV-1 surface glycoprotein interaction with MDDC induces release of type I IFN and IL-10 but no IL-12 or TNF-α.

In HSV-1 infection, type I IFN produced early in the infection by different cell types blocks viral replication and is thus important in viral resistance (Mittnacht S et al., 1988; Leib et al., 1999; Noisakran et al., 2000). Most cell types are able to secrete type I IFN. pDC, are known to secrete high levels of type I IFN in response to viruses, but with the appropriate stimulant myeloid DC can also induce secretion of large quantities of type I IFN, equivalent to that produced by pDC (Diebold SS et al., 2003). Therefore the type I IFN production was examined, monitoring secretion of IFNα protein and synthesis of IFNβ mRNA. Supernatants from MDDC stimulated for 18 hours with poly I:C (50 μg/ml), LPS (100 ng/ml), infected with HSV-1 or co-cultured with Cos-7 cell expressing all four entry glycoproteins were collected. Fig 4.4a shows that MDDC co-cultured with the four glycoproteins produced IFNα, as did MDDC co-cultured with whole virus, or with poly I:C. Co-culture with LPS did not result in release of IFNα at this time point. Neither MDDC cultured with mock transfected Cos-7 cells, nor Cos-7 cells alone, produced any detectable IFNα.

Following five hour stimulation as described above, MDDC were harvested and cDNA synthesized from the extracted RNA. IFNβ mRNA was detected in MDDC stimulated with LPS (100 ng/ml), poly I:C (50 μg/ml) and cells co-cultured with Cos-7 cells expressing the four glycoproteins (fig. 4.4b). No IFNβ was detected in unstimulated MDDC and only a minimal amount was detected in cells infected with HSV-1.

In contrast, neither HSV-1 virions nor the glycoproteins induced detectable IL-12p70 (fig. 4.5a), although the MDDC were capable of releasing large amounts of this

cytokine under appropriate stimulation with LPS. Both MDDC co-cultured with the four glycoproteins and with whole virus produced small amounts of IL-10. MDDC exposed to mock transfected Cos-7 cells, or Cos-7 cells on their own did not produce detectable IL-10 (fig. 4.5b). Neither the MDDC co-cultured with Cos-7 cells transfected with the four glycoproteins nor the MDDC exposed to HSV-1 virions produced significant levels of TNF-α (fig. 4.5c).

4.2.3. IRF7, but not IRF3 is translocated to the nucleus in MDDC in response to HSV-1 and the viral glycoproteins

To investigate the role of the IRF3 and IRF7 pathway in the induction of type I IFN in MDDC, cells were stimulated with different TLR ligands, infected with HSV-1 or co-cultured with transfected Cos-7 cells and the early (5 hours post-stimulation) nuclear translocation of IRF3 and IRF7 was measured by confocal microscopy. MDDC stimulated with imiquimod (a TLR7 ligand), CL075 (a TLR8 ligand), infected with HSV-1 or co-cultured with Cos-7 cells expressing the four entry glycoproteins all showed a significant nuclear translocation of IRF7, compared to unstimulated MDDC, which showed a nuclear to cytoplasmic ratio of less then one (fig. 4.6a). In contrast, the stimulated MDDC did not show nuclear translocation of phosphorylated IRF3 (fig. 4.6b).

Activation of NF-κB was also measured in both unstimulated and stimulated cells. MDDC stimulated with TNF-α, LPS, infected with HSV-1, co-cultured with viral glycoprotein, or mock transfected Cos-7 cells were harvested 1 hour post-stimulation. The activation of NF-κB was determined by measuring the level of intracellular phosphorylated NF-κB (fig. 4.7). Intracellular flow cytometry showed that LPS and

TNF-α both stimulate the phosphorylation of NF-kB as early as one hour following stimulation, whereas virus infected and glycoprotein stimulated MDDC did not.

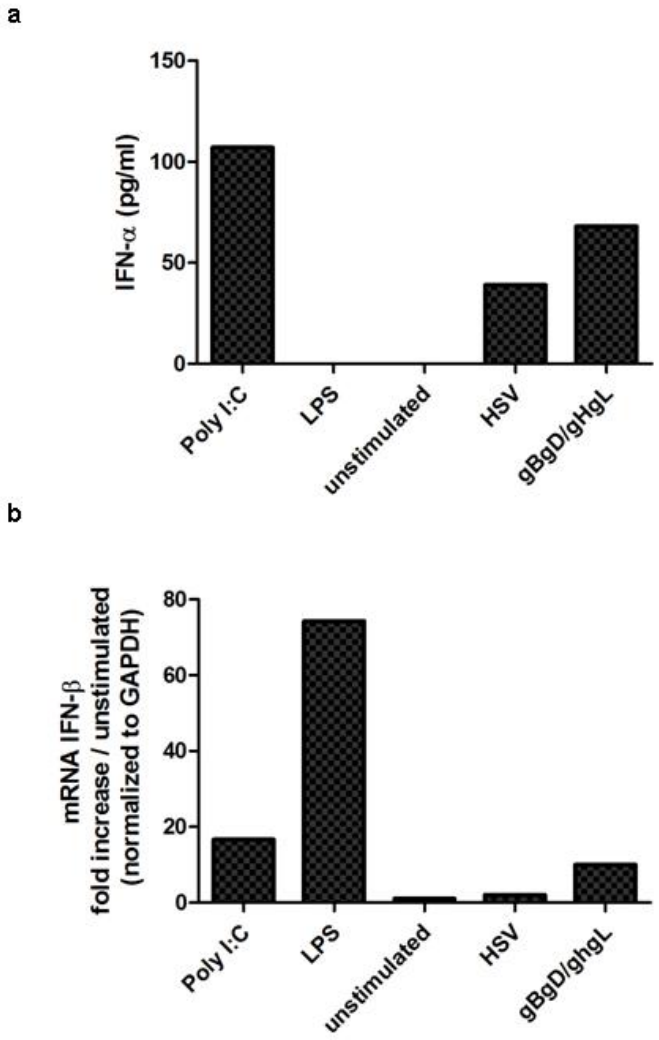


Figure 4.4: HSV-1 entry glycoproteins induce a type I IFN response.
a) MDDC were stimulated with poly I:C (50 μg/ml), LPS (100 ng/ml), HSV infected cells and cells co-cultured with transfected Cos-7 cells. Supernatants were collected 18-24 hours post-stimulation and IFN-α secretion was determined by ELISA. All ELISA experiments were performed in duplicates. A representative experiment from three independent experiments from three different donors is shown. b) RNA was extracted from MDDC stimulated as in figure a and cDNA was synthesized as described in Materials and Methods. IFN-β mRNA was measured using qPCR. Graph represents the ratio of IFN-β fold increase to unstimulated all normalized to GAPDH. All qPCR were preformed in duplicates. The figure shows one representative experiment from three independent experiments from three different donors.

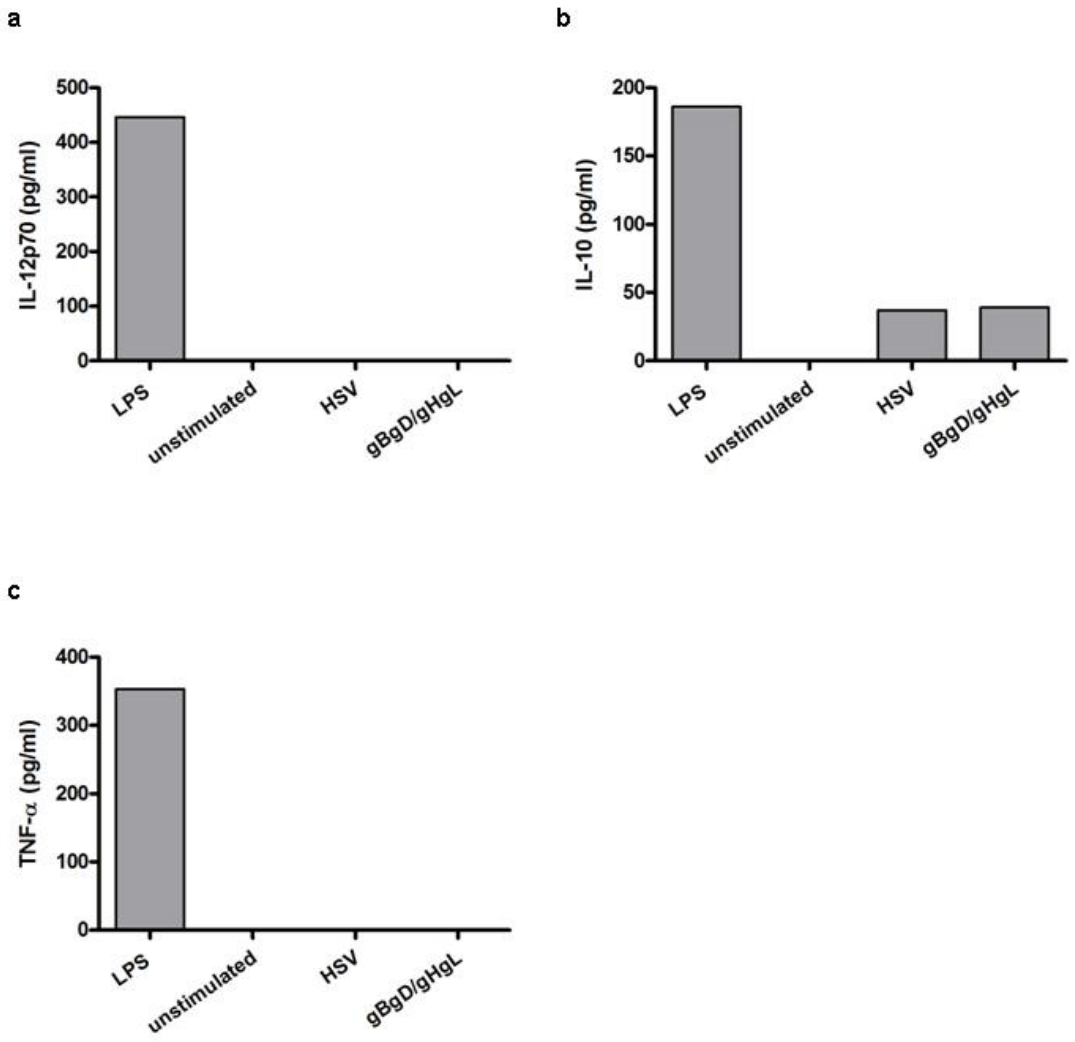


Figure 4.5: ELISA for IL-12p70, IL-10 and TNF-α. MDDC were stimulated with LPS(100 ng/ml), HSV infected or co-cultured with transfected Cos-7 cells. Supernatants were harvested 18-24 hours after stimulation and the cytokine secretion (IL-12p70, IL-10 and TNF-α) was determined by ELISA. All ELISA experiments were performed in duplicates. A representative experiment from at least three independent experiments is shown.

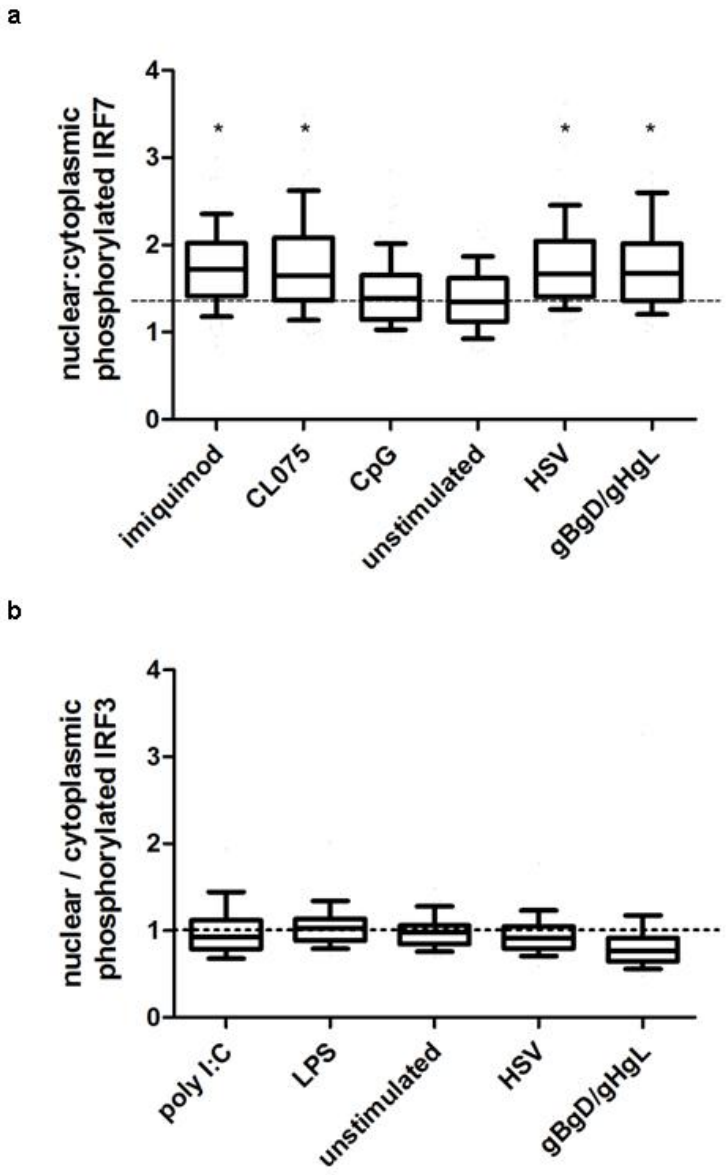


Figure 4.6: MDDC induce the nuclear translocation of phosphorylated IRF7, but not IRF3. Stimulated MDDC were fixed and permeabilized five hours post-stimulation and the nuclear:cytoplasmic ratio of a) IRF7 and b) IRF3 was quantified. Data points represent mean from at least seven separate high power field images from three different donors. Error bars represent the 5% and 95% confidence intervals. Horizontal dotted lines represent the nuclear:cytoplasmic ratio of 1. Statistical analysis was preformed using the one-way Anova test. * $P < 0.01$.

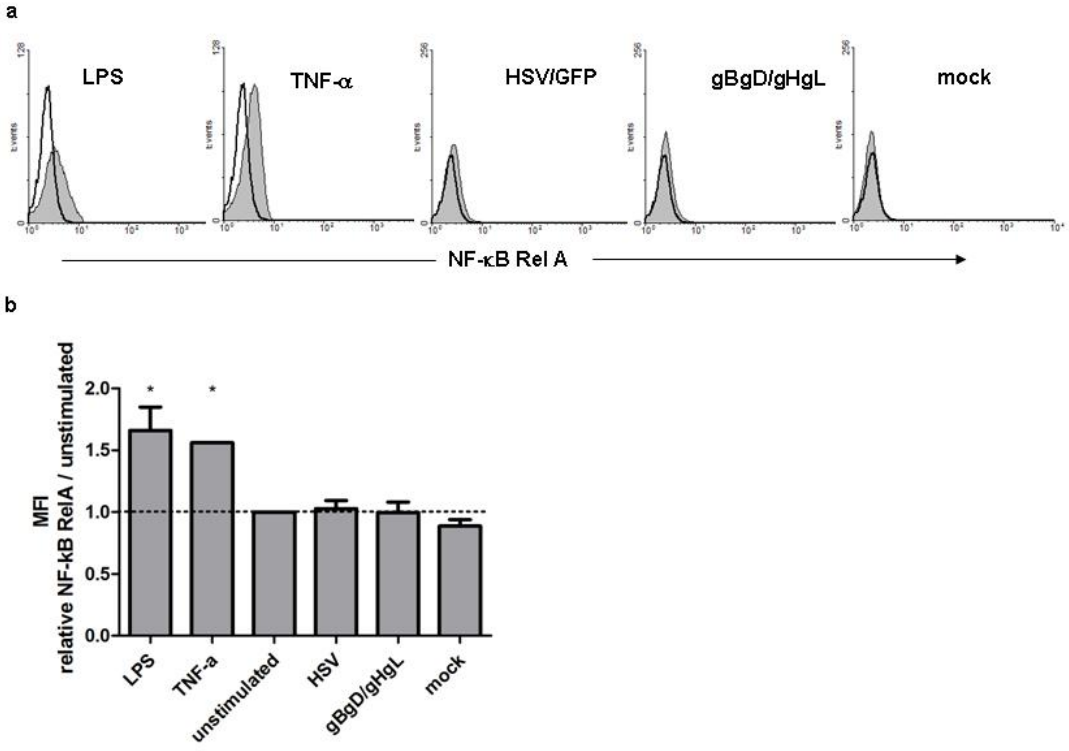


Figure 4.7: HSV-1 does not stimulate the early phosphorylation of NF-κB RelA. MDDC stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), HSV-1 infected, Cos-7 cells transfected with all four glycoproteins or mock transfected Cos-7 cells were fixed and permeabilized one hour post-stimulation. Phosphorylation of NF-kB RelA was measured by flow cytometry. a) Grey histograms represent the stimulated MDDC; open histograms represent the unstimulated MDDC. A representative example from three independent experiments from three different donors is shown. b) MFI showing the phosphorylated NF-kB relative to the native form. Data is from three independent experiments. Horizontal dotted line represents a NF-kB to unstimulated ratio of 1. Error bars represent the SEM. Statistical analysis was performed using the one-way Anova. * $P < 0.05$.

4.3. Discussion

In the previous chapter the HSV-1 glycoproteins necessary for viral entry have been shown to induce phenotypic maturation in MDDC. In this chapter, however, data is presented which shows that these glycoproteins failed to enhance MDDC activation of allogeneic T cells, and did not induce expression of the chemokine receptor CCR7. This finding suggests that, similar to whole virus, the glycoproteins induce "partial maturation", stimulating an innate antiviral response (i.e. type I interferon), but without enhancing T cell proliferation. Several examples of this type of partial maturation have been reported previously (Lutz and Schuler, 2002; Kleindienst et al., 2005; Verginis et al., 2005; Krathwohl et al., 2006; Braun et al., 2006; Bayry et al., 2007; Dulphy et al., 2007). In certain cases partial maturation has been associated with the induction of tolerance or regulatory T cells (Kleindienst et al., 2005; Verginis et al., 2005). However, here studies on representative T_H1 (IFN-γ), T_H2 (IL-13) and T_H17 (IL-17A) cytokines which could be secreted by the co-cultured T cells demonstrated that although the glycoprotein matured MDDC did produce a small amount of IL-13 (but no IL-17A), the main cytokine secreted was IFN-γ, implying a T_H1 response. Interestingly, T cells co-cultured with whole HSV-1 infected MDDC did not produce any IFN-γ. However, these T cells did produce minimal amount of IL-13, suggesting the virus skews the response towards a T_H2 response compared to a T_H1 response by the glycoproteins.

One of the key features of DC is their ability to produce a type I IFN in response to viral infections (Reviewed in Fitzgerald-Bocarsly and Feng, 2007). Indeed type I IFNs are key effector molecules of the innate anti-HSV-1 immune response (Leib et al., 1999; Noisakran et al., 2000; Casrouge et al., 2006) and release of IFNα induced maturation and viral resistance in neighbouring DC, which could mimic what happens *in vivo*

within and immediately around a zone of infection (Pollara et al., 2004b). Here the interaction of MDDC with glycoproteins and to a lesser extent infection by HSV-1, leads to the release of IFNα 18-24 hours post-stimulation. This release of IFNα is independent of viral DNA, although the possibility cannot be excluded that Cos-7 cells nucleic acid engulfed by the DC play a role.

With respect to IFNβ, qPCR for mRNA, showed that the glycoproteins, and to a lesser extent the whole virus, induce early IFNβ mRNA upregulation and presumably release of IFNβ immediately post-stimulation.

Several signaling pathways have been described for the initial production of type I IFN. Using a direct nuclear translocation assay, this study shows that the initial pathway activated following HSV-1 and the glycoprotein stimulation of MDDC is mediated via IRF7. Therefore, although the molecular pathway leading from glycoprotein recognition to DC response has not been characterised completely, the nature of this response suggests a model in which the glycoproteins initiate an initial IFNβ production upon interaction with DC; and this in turn upregulates the later and more long-lived production of IFNα via a positive feedforward loop. The low amount of detectable type I IFN stimulated by the whole virus, compared to the amount that the glycoproteins stimulated, may be attributed to one or more of several viral proteins known to be responsible for the evasion mechanisms (Mossman et al., 2000; Eidson et al., 2002; Harle et al., 2002; Melroe et al., 2004; Melroe et al., 2007).

With regard to other cytokines, MDDC stimulated with HSV-1 glycoproteins also produced small amounts of the anti-inflammatory cytokine IL-10, but did not produce any detectable IL-12. IL-12 is a key cytokine with an important role in the T_H1 response (Hsieh CS et al., 1993; Manetti R et al., 1993). However, recent studies

suggest that IL-12 is not an absolute requirement for the initial priming of cells towards T_H1, but is important for the subsequent expansion of these cells (Noble A et al., 2001). A similar high IL-10:IL-12 ratio has been found in recurrent HSV lesions *in vivo* (Mikloska Z et al., 1998). A previous study has reported IL-12 mRNA, but no IL-12 protein production in HSV-1 infected blood DC (Ghanekar et al., 1996). The lack of IL-12 response during HSV-1 infection can be attributed to the viral virion host shut off (vhs) protein which suppress the host's cellular protein synthesis (Smiley, 2004). However, the lack of response to Cos-7 transfected cells cannot be attributed to this protein. Down-regulation of IL-12 may also be the result of autocrine IL10 feedback, since Cos-7 cells expressing the four glycoproteins do stimulate IL-10 release, and IL-10 blocks transcription of both IL-12 subunit genes (D'Andrea A et al., 1993; Aste-Amezaga et al., 1998).

Neither the virus nor the viral glycoproteins induced any detectable immediate (one hour) NF-κB phosphorylation. This finding correlates with the lack of pro-inflammatory cytokine production observed in these cells, as several studies have suggested that rapid NF-κB phosphorylation is responsible for the induction of pro-inflammatory cytokine production (Kawai et al., 1999; Kaisho and Akira, 2001). Alternatively, this might be due to the sensitivity of the method applied, although phosphorylation could be detected in response to both LPS and TNFα.

4.4. Conclusion

In this chapter the functional consequences of the interaction between the viral glycoproteins and MDDC have been analyzed. The results demonstrate clearly that the four HSV-1 entry glycoproteins do not enhance the proliferation of co-cultured T cells, although they do elicit a T_H1 response. Furthermore, MDDC matured by the

glycoproteins produce a type I IFN necessary for an anti-viral response. This response was dependent on the initial IRF7 nuclear translocation.

CHAPTER 5 - HSV-1 ENTRY GLYCOPROTEIN INTERACTION WITH OTHER DC TYPES: LANGERHANS CELLS AND PLASMACYTOID DC

5.1. Introduction

The skin acts as a first line of defence against infection. In addition to a barrier function, there are two major functional compartments which can be elicited rapidly: an innate immune response, involving the recognition of the pathogen, and expression of pro-inflammatory cytokines and IFNs; and an "early warning" system, which attracts any adaptive immune response that has been primed previously (Meyer T et al., 2007).

Two subsets of DCs are found constitutively within the non-infected normal human skin. These are epidermal LC, present in the suprabasal layer of the epidermis, in tight contact with keratinocytes via E-cadherin-mediated adhesion, and DDC found within the dermis. These cells capture antigen and release pro-inflammatory cytokines in a non-antigen specific fashion and are responsible for the recruitment of more cells to the site of infection, including peripheral pDC.

In natural HSV-1 infection, the virus spreads by direct contact, entering the mucous membrane or damaged skin of the host. It has been suggested that upon infection, the virus interacts with both the epidermal LC and DDC, although there is no direct evidence in humans that HSV-1 infects either of these cell types *in vivo*.

Having shown that the interaction between MDDC, which have a phenotype broadly comparable to that which has been ascribed to DDC (Grassi et al., 1998), and viral entry glycoproteins induces a more mature phenotype, and a type I IFN response, in this chapter the *in vitro* interaction between HSV-1 and other forms of potential cutaneous DC has been investigated.

5.2. Results

5.2.1. Monocyte-derived Langerhans cells phenotype

MDLC were prepared as described in section 2.2.2 by the addition of TGF-β on day 0 and day 4 of monocyte differentiation. Similar to MDDC as shown in figure 3.1, day 7 MDLC cultures were a homogenous pure population of cells (data not shown). However, these cells were found to express E-cadherin, characteristic of LC (fig. 5.1a) and low levels of DC-SIGN while MDDC expressed high levels of DC-SIGN and little E-cadherin (fig. 5.1b).

To characterize the the key functional properties of the MDLC population further, expression of the maturation marker CD86 in response to different TLR ligands was compared between MDDC and MDLC (fig. 5.1c). Pam_3CSK4 (TLR2), poly I:C (TLR3), LPS (TLR4) and CL075 (TLR8) all induced significant upregulation of CD86 in MDDC, whereas only poly I:C and CL075 induced a significant upregulation of CD86 in MDLC. Neither cell population showed a maturation phenotype in response to either imiquimod (TLR7) or CpG oligodeoxynucleotides (TLR9). This is consistent with the reported lack of TLR7 and TLR9 on myeloid derived DC (Rozis et al., 2000; Takeuchi et al., 2003; van der Aar et al., 2007) and confirms that with respect to these two TLR, MDLC had the same phenotype as MDDC.

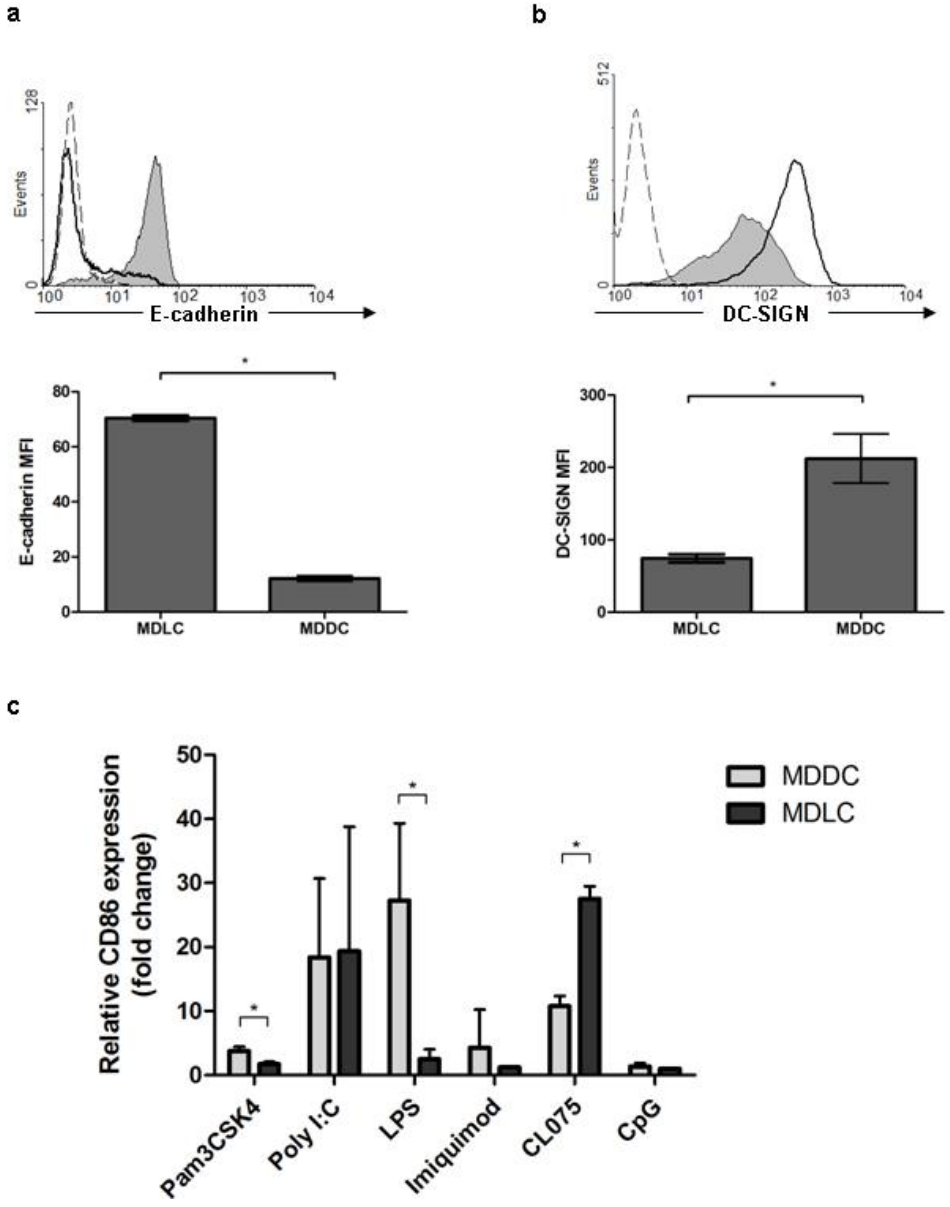


Figure 5.1: Comparative charachterization of MDDC and MDLC. MDDC and MDLC were prepared as describe in Materials and Methods and analyzed by flow cytometry. Expression of a) e-cadherin and b) DC-SIGN was measured. Empty profile shows staining of MDDC, filled profile shows staining of MDLC. b) The mean fluorescent intensity (MFI) level was compared between the two subsets (a and b lower histograms). Error bars represent the standard error of the mean (SEM). Statistical analysis was preformed using the Student's *t*-test. * $P < 0.05$. c) MDDC and MDLC were stimulated with different TLR ligands and the levels of CD86 were measured by flow cytometry. Graph represents the MFI relative to unstimulated cells. Error bars represent the SEM. Statistical analysis was preformed using the Student's *t*-test for each TLR ligand used. * $P < 0.05$. All experiments were performed three or more times.

5.2.2. Induction of MDLC maturation in response to HSV glycoproteins

To investigate the interaction of HSV-1 and the viral glycoproteins with LC, MDLC were either infected with virus or co-cultured with Cos-7 cells expressing all four glycoproteins. Viral infection (measured by expression of the GFP transgene) and expression of maturation phenotype was measured by flow cytometry (fig. 5.2). At a MOI of 1 pfu per cell, 55% of the MDLC were GFP positive (fig. 5.2a), similar to the reported infection efficiencies in MDDC (fig. 3.3a). MDLC infected with the virus also showed a maturation phenotype with upregulation of the maturation marker CD86 (fig. 5.2b).

Similar to the results obtained with MDDC, in MDLC Cos-7 cells expressing all four entry glycoproteins also induced upregulation of CD86 following an 18-24 hour co-culture. Mock transfected Cos-7 cells had no effect on CD86 expression (fig. 5.2b). However, CD83 and HLA-DR were only partially up-regulated in MDLC (fig. 5.2c). Thus, as with MDDC, HSV-1 is able to infect and induce phenotypic maturation of MDLC, and maturation requires only the four HSV-1 essential entry glycoproteins independent of any other viral components.

5.2.3. MDLC do not produce detectable type I IFN, IL-10, IL-12p70 or TNFα in response to maturation

The production of type I IFN by MDLC was also examined. MDLC were differentiated from the same donors as used for the measurement of MDDC cytokine production and stimulated with poly I:C (50 μg/ml), LPS (100 ng/ml), infected with HSV-1 or co-cultured with Cos-7 cell expressing all four entry glycoproteins. The supernatants were collected 18 hours post-stimulation. IFNα production was determined by ELISA. Only

poly I:C induced secretion of IFNα in MDLC, although quantitatively less than that produced by MDDC (fig. 5.3a). Neither MDLCs infected with HSV-1 nor co-cultured with Cos-7 cells expressing the four glycoproteins produced any detectable IFNα.

The upregulation of IFNβ mRNA was also determined in these cells. Five hours post-stimulation, RNA was extracted and cDNA synthesized as described in Materials and Methods. Compared to MDDC, where IFNβ mRNA was determined in the same reaction, MDLC did not upregulate any detectable IFNβ mRNA (fig. 5.3b). Together these data show MDLC exhibit significantly less capacity for antiviral type I IFN responses than MDDC.

Furthermore, MDLCs did not secrete IL-10 or IL-12p70 in response to either the virus or the viral glycoproteins, or in response to any of the TLR ligands tested. MDLC stimulated with Pam_3CSK4, poly I:C and LPS did, however, produce TNFα, although this was significantly lower than the amount induced by MDDC when stimulated with LPS (not shown). TNFα was not detected in either MDLC infected with HSV-1 or stimulated with Cos-7 expressing the four viral glycoproteins. Thus, although HSV-1 does infect MDLC, and MDLC responds to HSV-1 glycoproteins by upregulation of cell surface maturation markers, the MDLC did not respond in the same way as MDDC in terms of cytokine synthesis and release.

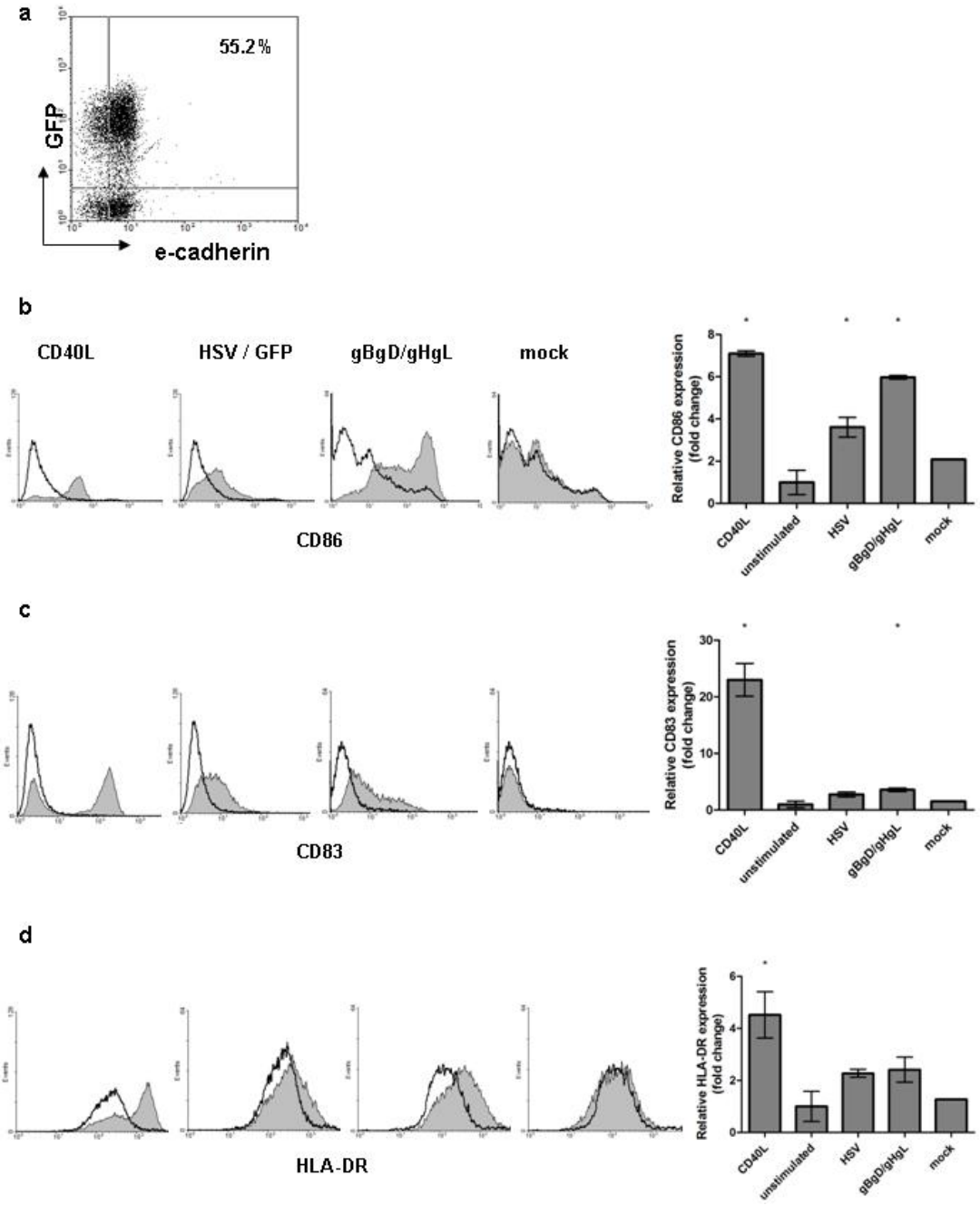


Figure 5.2: HSV-1 and Cos-7 cells expressing the four viral entry glycoproteins induce MDLC maturation. a) MDLC were infected with HSV-1/GFP MOI 1 and the percentage of cells expressing GFP was measured by flow cytometry. MDLC were infected with HSV-1/GFP or co-cultured with Cos-7 cells expressing the four viral entry glycoproteins and the level of b) CD86, c) CD83 and d) HLA-DR expression was measured. The small histograms represent HSV-1 infectivity as measured by GFP expression. The vertical dotted line indicates the mode of the distribution in unstimulated MDLC. All panels show one representative experiment from at least three independent experiments from three different donors. Right histograms show the relative MFI fold change from three independent experiments. Error bars represent standard error of the mean. Statistical analysis was performed using the one-way Anova test. * $P < 0.05$.

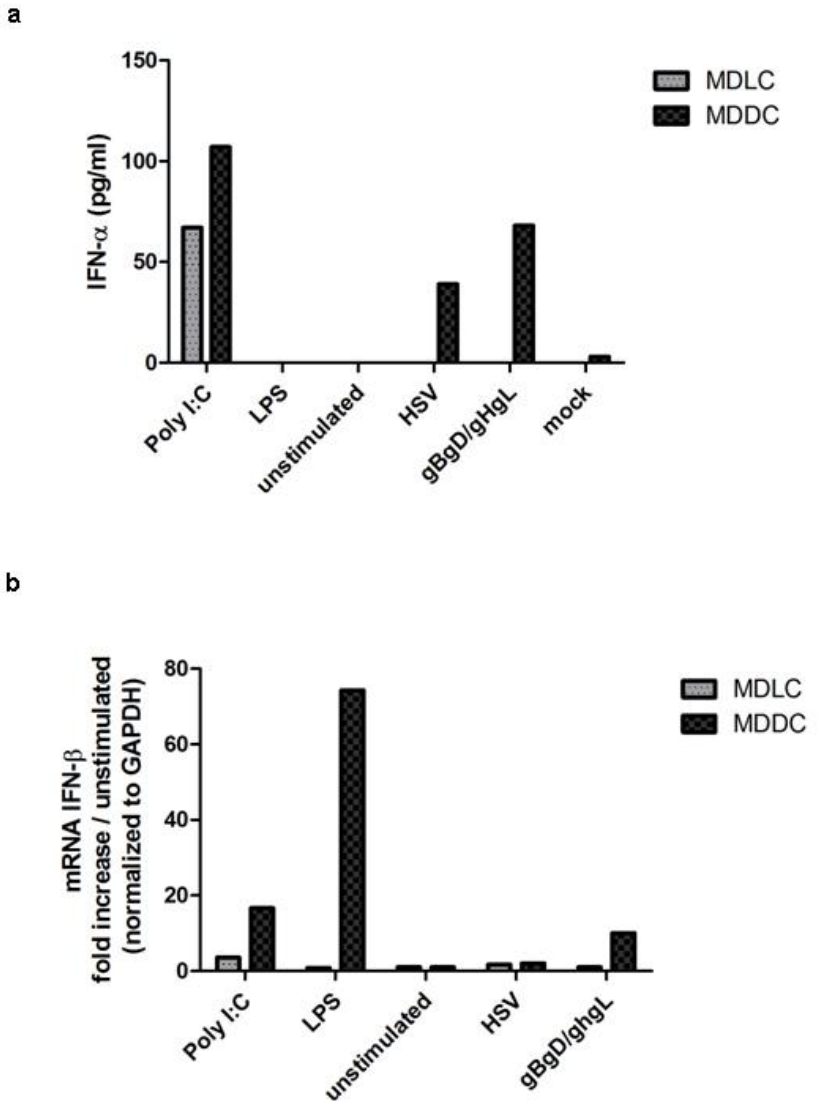


Figure 5.3: MDLC stimulation does not induce the production of type I IFN. a) MDDC and MDLC were stimulated with poly I:C or LPS, HSV infected, or co-cultured with transfected Cos-7 cells. Supernatants were harvested 18-24 hours post- stimulation and IFN-α secretion was determined by ELISA. All ELISA experiments were performed in duplicates. The figure shows one representative experiment from three independent experiments from three different donors. b) RNA was extracted from MDDC and MDLC stimulated as in figure a) and cDNA was synthesized as described in Materials and Methods. IFN-β mRNA was measured using qPCR. Graph represents the ratio of IFN-β fold increase to unstimulated, normalized to GAPDH. All qPCR were performed in duplicates. A representative experiment from three independent experiments from three different donors is shown.

5.2.4. MDLC do not induce the activation of T cells

HSV-1 virions and the viral glycoproteins do not enhance the ability of MDDC to induce proliferation of allogeneic T cells (see 2.2.1), but this has not been examined previously using MDLC as APCs. Thus, the ability of MDLC to activate allogeneic T cells was measured. In general, MDLC were less efficient that MDDC at stimulating T cells proliferation (fig. 5.4). Even when stimulated with LPS or poly I:C (fig. 5.4 top right panel), MDLC-induced proliferation was still significantly less than the proliferation of the same donor's T cells after co-culture with MDDC (fig. 5.4 top left panel).

To consolidate these findings further, the cytokines secreted by the MDLC stimulated T cells (IFN-γ, IL-13 and IL-17A) were also measured by ELISA as described for MDDC. T cells co-cultured with MDLC did not produce detectable cytokines at 24 hours. After 6 days of culture, the T cells co-cultured with poly I:C and viral glycoproteins stimulated MDLC also secreted IFN-γ, although the amount of this cytokine measured was close to the ELISA sensitivity threshold (fig. 2.1), and was less than that induced by T cells co-cultured with MDDC ($p < 0.01$) (fig. 5.5a). T cells co-cultured with MDLC matured by LPS, poly I:C and the viral glycoproteins also produced low amounts of IL-13. None of the MDLC stimulated T cells produced any IL-17A.

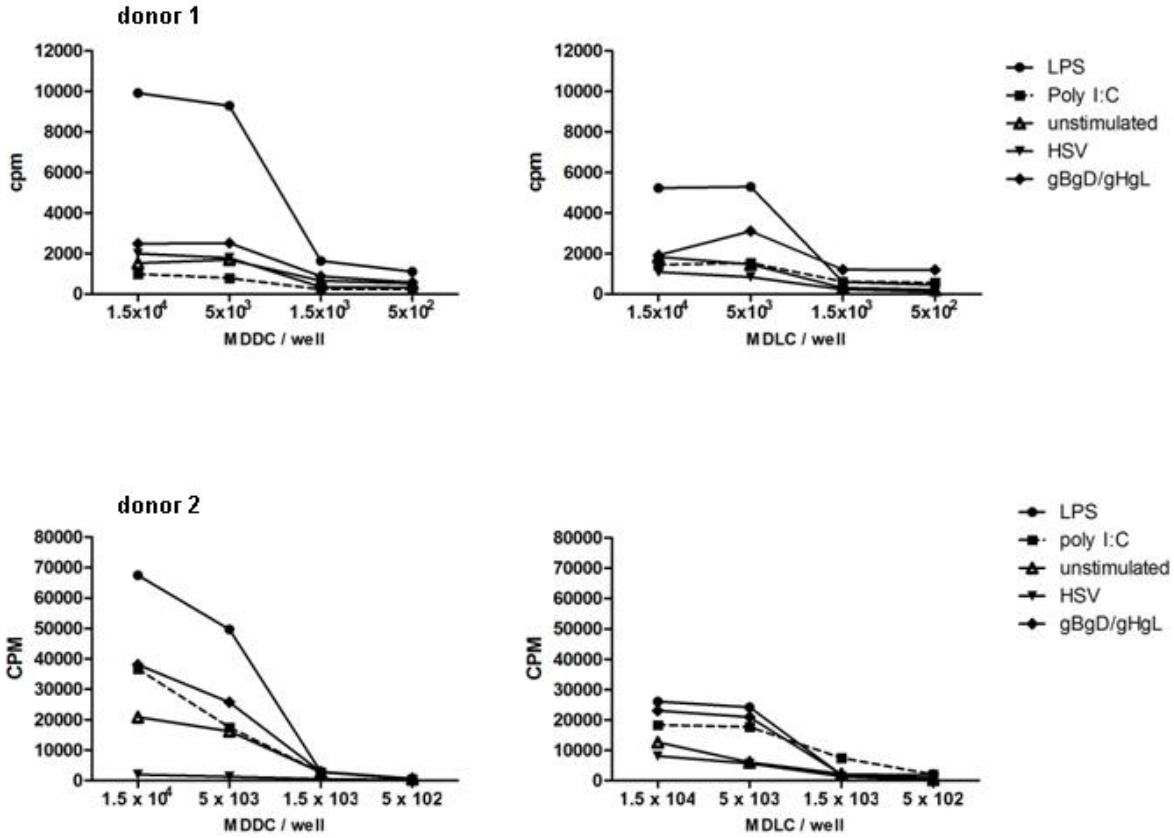


Figure 5.4: MDLC do not induce the activation of allogeneic T cells. MDDC and MDLC co-cultured with Cos-7 cells expressing the four glycoproteins, mock transfected, or infected with HSV-1, stimulated with LPS, poly I:C or unstimulated were used to stimulate allogeneic T cells proliferation. Figure shows the mean counts per minute (cpm) of triplicate wells of T cells in a DC dose response. Experiments from two different donors representative of three independent experiments from three different donors are shown.

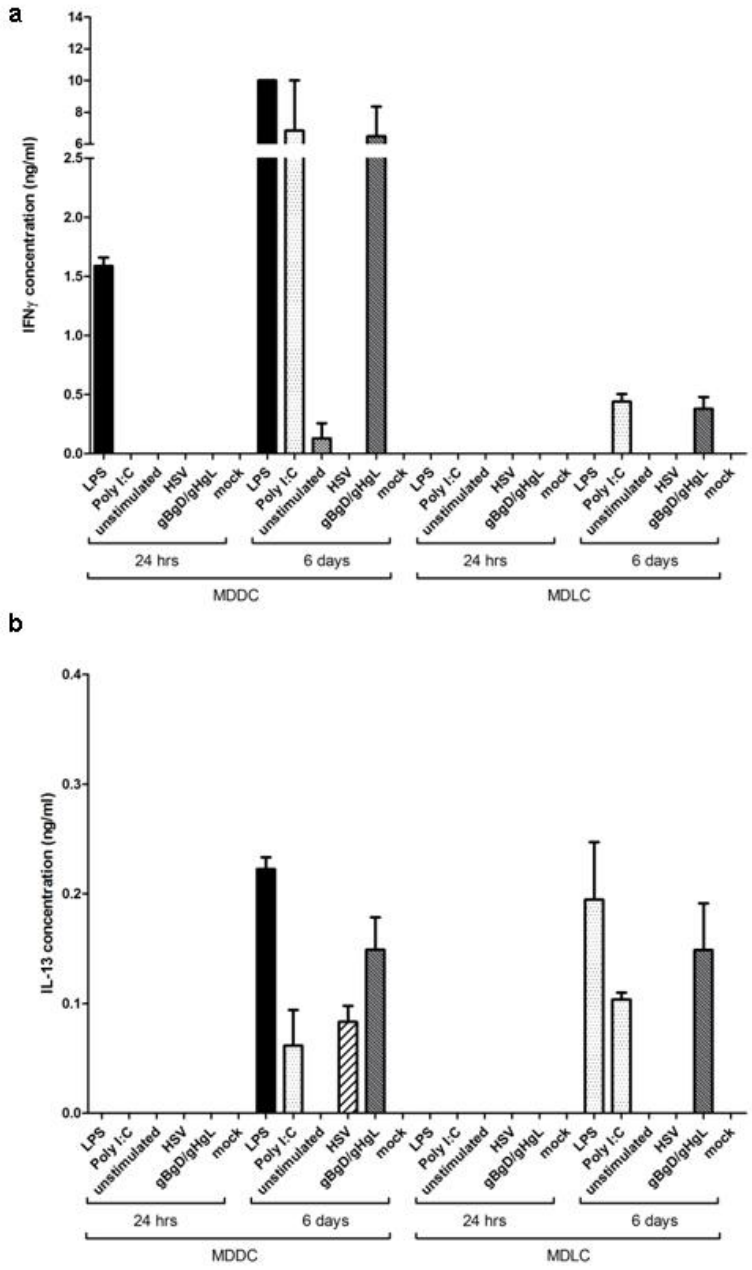


Figure 5.5: ELISA for T cell T_H1 and T_H2 cytokines. MDDC and MDLC were stimulated as described in figure 5.4 and cultured with allogeneic T cells. Supernatants were collected at 24 hours and 6 days post-co-culture. Secreted a) IFN-γ and b) IL-13 were determined by ELISA. All ELISA experiments were performed in duplicates. Error bars represent standard error of the mean. All experiments were performed three times from three independent donors.

5.2.5. HSV-1 glycoproteins do not induce maturation in peripheral plasmacytoid DC

pDC are a distinct subtype of DC expressing TLR-9 with a potent ability to produce high levels of type I IFN in response to viral infection (Siegal et al., 1999; Asselin-Paturel et al., 2001; Colonna et al., 2002). These cells are known to express CD303 and CD304 on their surface, and therefore these markers were used to isolate them from PBMC (fig. 5.6).

HSV-1 virion induced upregulation of the maturation marker CD80 in pDC (fig. 5.7b). On these cells CD83 was not upregulated. Interestingly, only 4% of the gated cells (5% of total pDC) expressed GFP (fig. 5.7a). Neither the four glycoproteins, nor the single glycoproteins induced maturation (fig. 5.7c).

Supernatants from pDC co-cultured with Cos-7 cells expressing the four glycoproteins, mock transfected Cos-7 cells, HSV-1 infected pDC and CpG stimulated pDC were used for the measurement of IFNα and TNF-α. In accordance with the flow cytometry results, both the CpG stimulated and the HSV-1 infected pDC produced both cytokines, while the pDC co-cultured with the mock transfected and the four glycoproteins transfected Cos-7 cells did not (fig. 5.8).

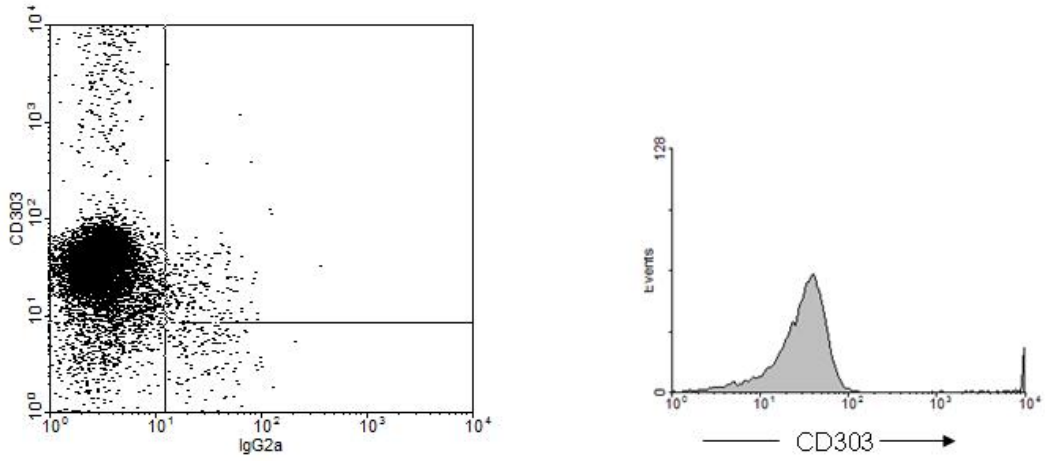


Figure 5.6: Peripheral plasmacytoid DC phenotype. pDC were isolated from PBMC by immunomagnetic cell sorting for CD304. They were examined for expression of CD303. A homogenous pure population of pDC is shown.

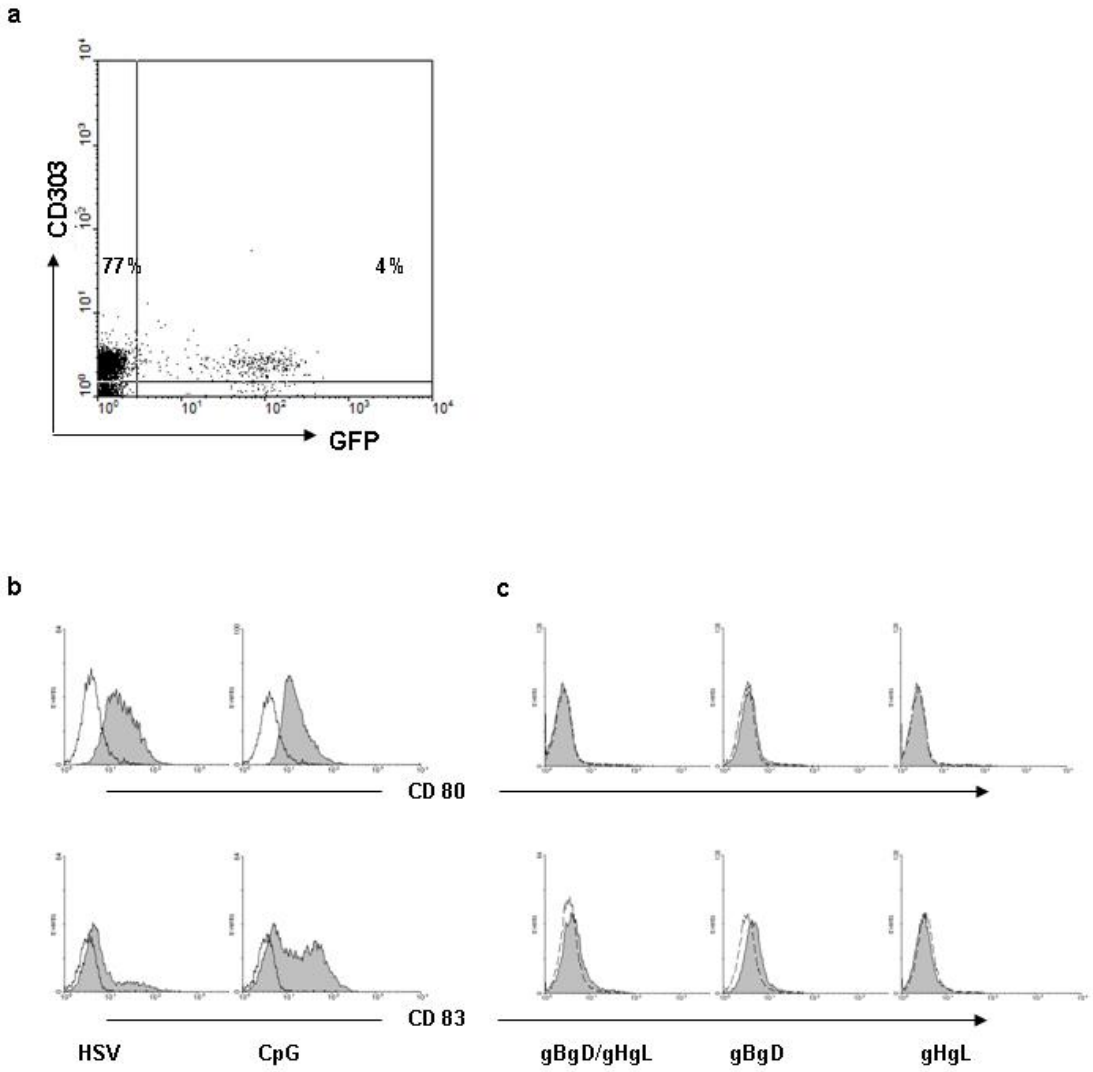


Figure 5.7: HSV-1 entry glycoproteins do not induce pDC maturation. a) pDC infected with HSV-1/GFP and the percentage of cells expressing GFP was measured by flow cytometry. b) pDC were infected with HSV-1 or stimulated with CpG (and the expression of CD80 and CD83 was measured by flow-cytometry. Empty histograms represent the unstimulated pDC. c) pDC were co-cultured with Cos-7 cells expressing the four HSV-1 entry glycoproteins or single glycoproteins and the expression of CD80 and CD83 was measured. Empty histograms represent pDC co-cultured with mock transfected Cos-7 cells. A representative of two independent experiments from two different donors is shown.

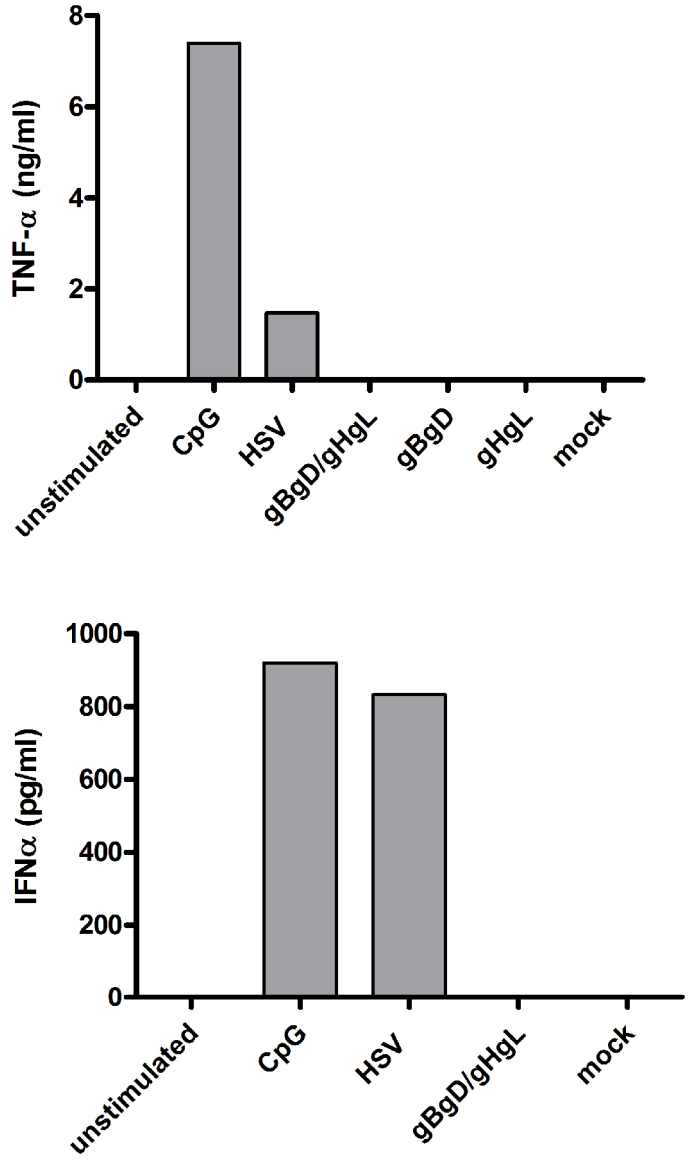


Figure 5.8: HSV-1 entry glycoproteins do not induce pDC cytokine secretion. pDC were stimulated with CpG (5 μg/ml), HSV-1 infected, co-cultured with Cos-7 transfected with all four glycoproteins, Cos-7 cells transfected with single glycoproteins or Cos-7 cells mock transfected. Supernatants were collected 24 hours post-stimulation and TNF-α and IFN-α were measured by ELISA. All ELISA experiments were performed in duplicates. A representative experiment from at least two independent experiments is shown.

5.3. Discussion

The predominant site of HSV-1 primary infection is in the skin and mucosal surfaces. Therefore, in order to understand both immune defence against the virus, and associated immunopathology, it is important to identify and analyze the APCs that are found at these sites that might be implicated in the early responses to the virus. Therefore in this chapter the function of MDLC, as representative of epidermal LC, and of pDC, were studied in response to HSV-1 and the viral glycoproteins.

Most previous functional studies on LC, as the prototype of skin DC, and their responses to pathogens such as HSV-1, have focused primarily on cells isolated from animal models (Geissmann et al., 1998; Nunez et al., 2004). This is due, at least partly, to the fact that direct *ex-vivo* isolation of LC from human skin is associated with significant spontaneous maturation, possibly due to the migration step that is used most often when isolating these cells from skin explants (Tchou et al., 2003). In a single preliminary study, we attempted to isolate epidermal LC directly from the skin by migration, but similar to Tchou's studies, we found the "unstimulated" LC to be in a state of maturation (Appendix B).

A modified MDLC protocol, which generates cells with distinguishing features that discriminate them from conventional MDDC, has been described (Geissmann et al., 1998). Therefore, in this study we focused on these MDLC, and compared them with MDDC.

Like LC, DDC are a probable site of early contact and response to HSV-1; and, like LC, *ex vivo* derived DDC are difficult to isolate and investigate. Thus in the first part of the study we were concerned not only with characterization of the MDLC, but also with confirming that the MDDC did resemble what is known about DDC phenotype, and about their core functions, as exemplified by responses to TLR ligands. The key

features which discriminate between the different DC forms are summarized in Table 5.1. Both the cell surface phenotype (DC-SIGN^{low} and E-cadherin^{high} for MDLC, DC-SIGN^{high} and E-cadherin^{low} for MDDC), and the functional TLR expression (TLR2^{low}, TLR3, TLR4^{low} and TLR8 for MDLC; TLR2, TLR3, TLR4 and TLR8 for MDDC) was consistent with that reported for LC and DDC respectively (van der Aar et al., 2007; Ebner et al., 2004; Teunissen, 2005; Flacher et al., 2006). Other studies have shown different TLR expression in blood-derived LC-like cells (Renn CN et al., 2006; Rozis G et al., 2008), but these LC-like cells were derived from CD34+ cord blood cells, and their TLR expression was different from that of *ex-vivo* epidermal LC.

Next we compared the two cell types with respect to their response to HSV-1, the four essential viral entry glycoproteins, gB, gD and the heterodimer gHgL. In previous studies (Pollara et al., 2003) and in the current work (chapter three and four) both HSV-1 and the these glycoproteins expressed on the surface of transfected Cos-7 cells act as a novel PAMP, inducing maturation and partial activation of MDDC. In this study we extended these studies to show that MDLC also respond to HSV-1 and the glycoproteins, by upregulating surface markers expression, suggesting that these APCs are also capable of recognizing the HSV-1 surface.

One of the key features of DC is their ability to produce a type I IFN response to viral infections (Fitzgerald-Bocarsly and Feng, 2007). Type I IFNs are key effector molecules of the innate anti-HSV-1 immune response (Leib et al., 1999; Noisakran et al., 2000; Casrouge et al., 2006). Here, compared to MDDC, which produce both IFNα and IFNβ in response to TLR stimulation, and to both the virus and the viral glycoproteins, MDLC do not show a type I response.

The comparative role of MDDC and MDLC in the activation of T cells was also examined. Our previous studies demonstrated that neither HSV-1 nor viral glycoproteins enhanced the ability of MDDC to stimulate T cell proliferation (section 4.2.1), and indeed infection with HSV-1 was associated with a reduction in stimulating activity. A similar general pattern was now observed in MDLC, although in general MDLC were weaker activators of T cells than MDDC.

Finally, the T_H1 and T_H2 cytokine (effector) responses of the T cells to MDDC and MDLC, with and without pre-stimulation were examined. Supernatants from T cells co-cultured with stimulated DC cells were collected at 24 hours and 6 days to measure both immediate and late T cell cytokine secretion. MDLC were poor stimulators of IFNγ even after stimulation, but responded to HSV glycoproteins by producing IL-13 in response to LPS, poly I:C and the glycoproteins. There was no response to HSV-1.

The data presented in this chapter demonstrate differences between MDDC and MDLC in the response to HSV and the viral glycoproteins. Although MDLC can be infected by the virus and demonstrate a maturation phenotype in response to the viral glycoproteins, these cells are not activated by the virus or the glycoproteins. This is consistent with previous studies, particularly with HSV-2 in a mouse model, which suggested that LC have a less direct role in the immune response compared to the other DC forms (Allan et al., 2003; Zhao et al., 2003; Belz et al., 2004; Lemos et al., 2004). The present study provides useful confirmation that this is true for human immune responses to HSV-1. This question is discussed in more detail below in section 6.3.

	MDDC	MDLC	LC [1]	DDC [2]
DC-SIGN	high	low	low	high
E-cadherin	low	high	high	low
TLR2	high	low	low	high
TLR3	high	high	high	high
TLR4	high	low	low	high
TLR7	NE	NE	NE	low
TLR8	high	high	Low	high
TLR9	NE	NE	NE	NE

NE = not expressed

[1] (Teunissen, 2005; Flacher et al., 2006; van der Aar et al., 2007)
[2] (Teunissen, 2005; van der Aar et al., 2007)

Table 5.1: Characterization of MDDC and MDLC compared to ex-vivo LC and DDC.

With respect to pDC (Asselin-Paturel et al., 2001), which were also investigated here, high levels of IFN-α and TNF-α were detectable in pDC stimulated with CpG, a TLR-9 ligand, and in pDC infected with the HSV-1 virions, although the percentage of pDC infected with HSV-1 was low compared to the percentage infectivity seen in MDDC and MDLC. HSV-1 DNA has been shown to carry CpG motifs, hence rendering the virus to be highly immunostimulatory via TLR-9 (Lundberg et al., 2003). These results are consistent with studies on murine pDC showing TLR-9-dependent recognition of HSV DNA (Lund et al., 2003; Hochrein et al., 2004; Krug et al., 2004). However, no cytokine production was detected in pDC co-cultured with the Cos-7 cells expressing the four glycoproteins, suggesting that the viral DNA is responsible for the TLR9 activation in pDC and not the viral entry glycoproteins.

5.4. Conclusion

In this chapter the interaction of the virus and the viral entry glycoproteins with other subtypes of DC has been investigated. The results demonstrate clearly that MDLC, thought to represent human epidermal LC, mature in response to the virus and the Cos-7 cells expressing the four entry glycoproteins. However, in contrast to MDDC, these cells do not induce a type I IFN or a T_H1 response, and therefore, in this form they may not play a major role in the immediate response to primary HSV-1 infection. In addition, unlike MDDC, interaction between virus and pDC demonstrated clearly that the viral glycoproteins do not activate these cells.

CHAPTER 6 – GENERAL DISCUSSION

6.1. Introduction

Previous studies have suggested a model which attempts to explain how DCs overcome the ability of infective HSV-1 to inhibit antigen presentation (Pollara, 2005). In this model, myeloid DC infected with HSV-1 produce sufficient IFNα to prime bystander uninfected DC, and to induce maturation and migration of these DC, which cross-present antigen from dying infected cells. In this thesis this work has been extended. One of the ligands of HSV-1 that elicit the activation of the DC and induce the signalling changes that bring about type I IFN secretion has been identified. The main emphasis of the study was on the viral entry glycoproteins (gB, gD and gHgL) only, and how they interact with the DC. Further studies should also look at other surface glycoproteins and their interactions with DC. In addition LC and pDC models were examined in order to develop a more comprehensive model of the innate immune response to HSV-1.

6.2. HSV-1 entry glycoproteins as inducers for DC maturation

HSV-1 entry occurs when extracellular virions attach to the cells surface via gC and gB, and then bind to a gD receptor activating the membrane fusion machinery comprised of gB, and the heterodimer gHgL (Campadelli-Fiume G et al., 2000; Spear, 2004; Reske A et al., 2007) (fig. 1.2). After this initial step, concomitant virus binding on the cell surface and membrane fusion mediated by these glycoproteins is thought to activate intracellular immunosignalling processes. Chapter 3 demonstrates clearly that these entry glycoproteins function as novel DC maturation inducers, independent of other viral components, both in the maturation of MDDC and in secretion of type I IFN. Their function seems to require the co-operative interaction of all four glycoproteins, acting together presumably as a complex. This complex formation is a known phenomenon in HSV and several studies have shown the importance of such complex formation in the

glycoprotein function (Avitabile et al., 2007; Atanasiu et al., 2007). However, this is the first study to show the importance of the glycoprotein complex in the maturation of DC.

Several receptors have been shown to associate with the HSV virions in entry and cell signalling pathways. HVEM and nectin-1 are two receptors expressed on cells, including DC, which are known to interact with the viral glycoprotein D, and to be important for viral entry into cells. Studies on cell lines expressing HVEM have also demonstrated that interaction of gD with this receptor can lead to the activation of the NF-kB signaling pathway (Sciortino MT et al., 2008). In this study, however, the direct interaction between gD and HVEM or nectin-1 on the MDDC surface does not appear to be essential or sufficient for initiating DC maturation. Other receptors associated with HSV-1 signalling, TLR2 and TLR9 where also shown not to be essential in the maturation of DC by the glycoproteins, suggesting another receptor(s) must play a role (discussed further in section 6.5).

6.3. Direct and indirect interactions in primary HSV-1 – DC interactions

In chapter 4 and 5, the biological consequences and the interaction of the viral glycoproteins with different DC subsets were studied. The results clearly demonstrate that MDDC infected directly with HSV-1 are inhibited in their ability to induce an effective anti-viral immune response. Although HSV-1 infected MDDC produce IFNα, this response was lower than that produced by HSV-1 infected pDC. MDDC which are exposed to viral glycoproteins, are primed to induce an immediate type I IFN response, which may not only contribute to the anti-viral state, but also contribute to stimulating a strong protective T_H1 response. In contrast, MDLC were poor stimulators of type I IFN T_H1 responses, but did respond to the viral glycoproteins, stimulating the production of IL-13. pDC did not respond at all to the viral glycoproteins.

Together these results support a model, in which the major stimulation of protective T cell immunity to HSV-1 could be via DDC. These cells could be exposed to viral glycoproteins released from lytic keratinocytes infections, from infected fibroblasts in the dermis, or from infected LC that are migrating through the dermis. Ultimately they could pick up and cross-present the glycoproteins, and at the same time are capable of producing an immediate type I IFN response. In addition to its anti-viral activity, type I IFN may also be an important danger signal detected by bystander uninfected DC, inducing activation of more APCs (Luft et al., 2002; Pollara et al., 2004a). Possibly pDC recruited to the site of infection can produce a stronger type I IFN response, induced by HSV DNA. In contrast, any DC infected with virus, or expressing glycoproteins, and any LC similarly altered in a similar fashion are inhibited in their T_H1 stimulating ability, but may drive a T_H2 response which may be relevant to the high production of antibody that has been observed in infected individuals. Alternatively, however, the findings are also consistent with a model where LC infected with HSV-1 in the epidermis may transport antigen to the LNs, and then transfer the antigen to LN resident DC for antigen presentation and T cell priming (Allan et al., 2003) (fig. 6.1). Irrespective of the in vivo balance between these two models, these findings add further support to the hypothesis explored previously (Pollara, 2005), i.e. that it is the "bystander" activated DC, not the infected or directly stimulated form, that act as the major APC in viral infection.

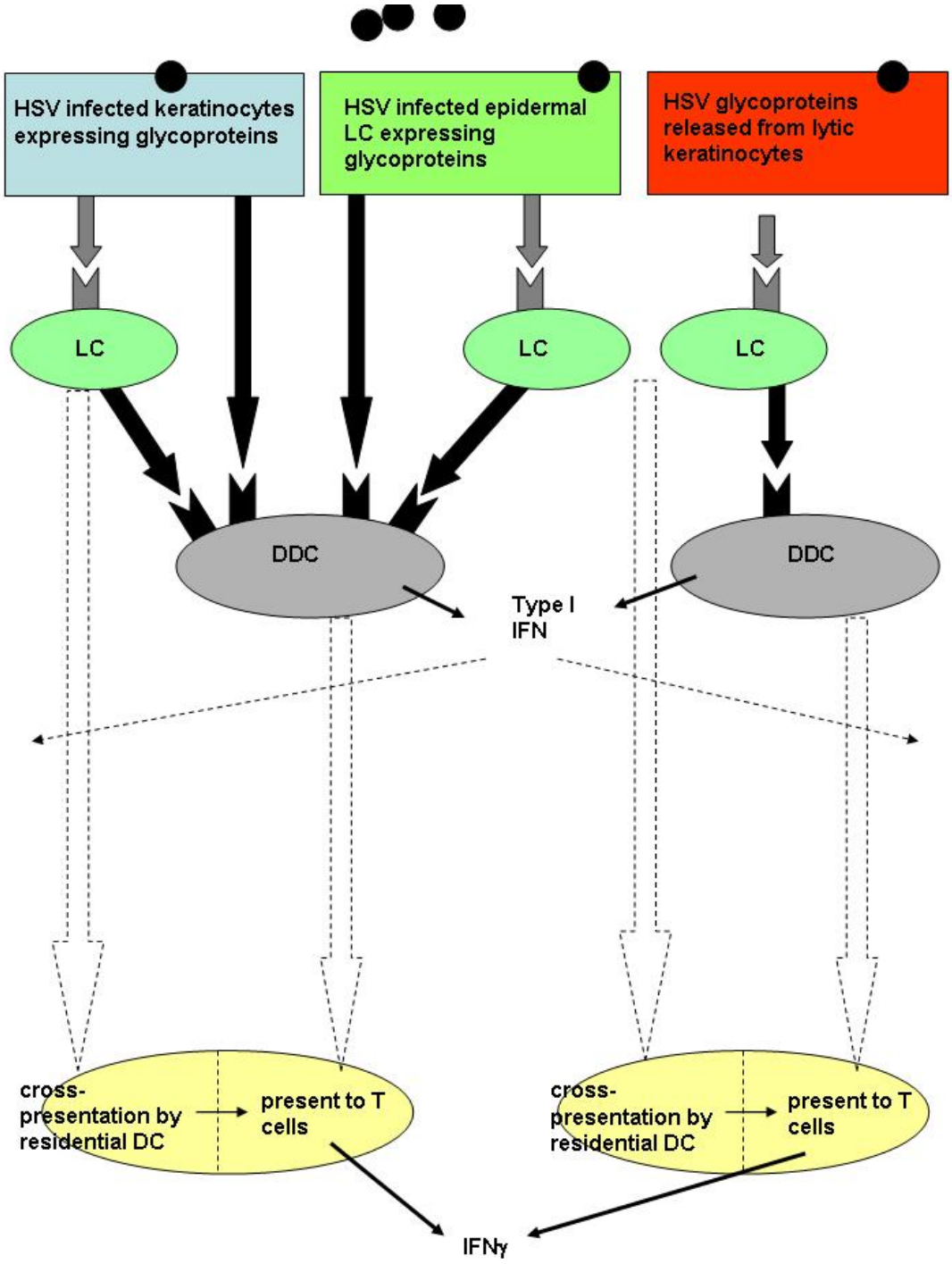


Figure 6.1: Direct and indirect interaction in HSV-1 – DC interactions. Following primary HSV-1 infection, viral glycoproteins expressed on infected keratinocytes, infected LC are cross-presented (filled black arrows) by DDC. Ultimately, these cells migrate (dotted arrows) to the lymph nodes to present the viral antigen to T cells which produce a IFNγ T_H1 response. At the same time, these cells produce sufficient type I IFN to recruit additional myeloid DC and pDC, where they produce additional type I IFN. Alternatively, glycoproteins expressed on infected keratinocytes, infected epidermal LC and/or released from lytic cells are cross-presented (filled grey arrows) by non-infected LC which migrate to the lymph nodes transferring the antigens to dermal DC or to LN resident DC for antigen presentation and T cell priming.

6.4. Clinical implications

6.4.1. Development of an HSV vaccine

HSV infection can result in a number of severe complications, especially in neonates and the immunocompromised (Stewart JA et al., 1995; Jacobs, 1998). Additional complications include stromal keratitis, the leading cause of infectious blindness (Liesegang, 2001). More recently, epidemiological studies suggest that in the presence of HSV infection, specifically HSV-2, the susceptibility and sexual transmission of HIV infection is increased. Furthermore, HSV-2 infection may be associated with an increase in blood HIV verimia (Schacker et al., 2002; Corey L et al., 2004; Rebbapragada et al., 2007). Hence, an effective vaccine would be beneficial both in controlling HSV morbidity and mortality, as well as beneficial in the control of HIV spread.

Over the years several approaches to the design of an effective HSV vaccine have been explored, from live attenuated virus to the use of recombinant viral subunits (Stanberry LR, 2004; Koelle DM, 2006). However, to date, all have failed to reach the endpoint of protection from infection in clinical trials. In this section the focus is on the development of glycoprotein subunit vaccines, which are likely to provide a safer alternative to live virus.

Several past efforts have demonstrated promising results in animal models using recombinant glycoprotein vaccines, specifically gB and gD, two essential glycoproteins that are cross-reactive between HSV-1 and HSV-2 (Peng et al., 1998b; Ghiasi et al., 1996; Manservigi et al., 2005). Recombinant HSV-1 gD induced an antibody response in Japanese macaques, which could be increased following intracutaneous boosting (Hirano et al., 2002). A variable T cell response was also detected. Recombinant gB and gD proteins have also been used as candidate subunit vaccine in human subjects. Thus

far, a combined HSV-2 recombinant gD-gB subunit vaccine with adjuvant, despite inducing high titres of specific antibodies, provided only a transient protection to infection (Corey et al., 1999). Another recombinant gD vaccine given with a more effective adjuvant (AS04), showed resistance to genital herpes in women who were seronegative at the time of vaccination to both HSV-1 and HSV-2, however failed in the protection of both men (regardless of their HSV serological status at time of vaccination) and women who were seropositive to HSV-1 (Stanberry et al., 2002). Further studies in animal models demonstrated that the effectivness of the vaccine was dependent on the adjuvant which skews towards a strong T_H1 response (Bourne et al., 2003).

Recently in addition to the recombinant protein vaccines, DNA plasmids expressing HSV glycoproteins have been considered as potential vaccines (McClements WL et al., 1996; McClements WL et al., 1997; Flo, 2003). DNA vaccination has significant advantages over other alternative immunization methods, because it is capable of inducing both arms of the adaptive immune response based upon very efficient delivery of heterologous proteins to APC of either transfected or infected cells, hence ensuring the transmission of a full activation signal to T cells. In addition, viral antigens may be administered together with genes encoding cytokines and chemokines which further enhance the function of the APC (Lee et al., 2003a; Lee et al., 2003b). The potency of immunity can be amplified further by the combination of DNA vaccines with fowlpoxvirus or modified vaccinia virus expressing similar antigens in a “prime-boost” strategy (Ramshaw and Ramsay, 2000).

Thus far, however, the design of an effective HSV vaccine based on one, or a combination of two, glycoproteins has been unsuccessful. In light of the data presented

in this thesis demonstrating the optimum requirement for a complex of all four HSV entry glycoproteins for DC stimulation, it could be that the immune stimulation achieved is sub-optimal, and that a vaccine containing the complex of all four entry glycoproteins may be more successful. However, it should be noted that the reconstitution of a functional entry/activation complex in a cell free system has not yet been achieved.

6.4.2. HSV as a gene delivery vector to DC

Initially HSV was suggested as an ideal vector for gene delivery to the nervous system (Lachmann and Efstathiou S, 1997). Its ability to infect several cell types efficiently and express multiple engineered genes suggested it to be a suitable candidate for the delivery of exogenous genes to DC as well (Coffin et al., 1998). However, in order to achieve an effective gene delivery, several viral factors disrupting DC function need to be removed. These include ICP47, which is thought to interfere with peptide translocation via TAP and subsequent loading on to MHC class I; gB, which may interfere with the loading of MHC class II; vhs, which may be responsible for changes in DC function by a mechanism involving the degradation of cellular mRNA; ICP27, which may be responsible for the shutdown of host protein synthesis and blocking of STAT-1, resulting in inhibition of type I IFN production; ICP4, which may be responsible for host protein synthesis shut off and to the inhibition of cytokine production; and ICP0, which may inhibit nuclear accumulation of IRF3, hence interfering with type I IFN signalling. Early studies have suggested that it is possible to produce an HSV-1 vector able to efficiently transduce DC which also allows the activation of DC necessary for an effective immune response (Samady et al., 2003).

These studies have not been followed up, however, and further studies are necessary in order to establish an efficient HSV-1 vector.

Another method of gene delivery that has gained much attention in recent years is the use of viral amplicon vectors – defective, non-integrative, helper-dependent vectors, derived from HSV-1. These viral particles are identical to the wild-type in terms of structure, but carry an amplicon plasmid carrying the transgene of interest instead of the viral genome. Recent works have looked at this approach for tumour therapy and the treatment of neuro-degenerative disorders (Cuchet D et al., 2007).

The data presented here, demonstrating the efficiency of the HSV-1 surface glycoproteins in inducing an immune response in DC, raises the possibility that other delivery systems should be considered. One example of this could be virus-like particles (VLP), which are empty non-infectious viral capsids with a similar morphology to the virus from which they have been derived (Petry H et al., 2003). Although most recent studies into VLP technology have looked at vaccine development, some research groups have demonstrated that VLP could also represent a useful gene therapy delivery system (Malboeuf CM et al., 2007).

6.5. Future work

This project follows from previous work which tried to solve some of the puzzle of the interaction between HSV-1 and DC (Pollara, 2005), and, as well as offering some answers, has generated many more unresolved questions that would be of interest.

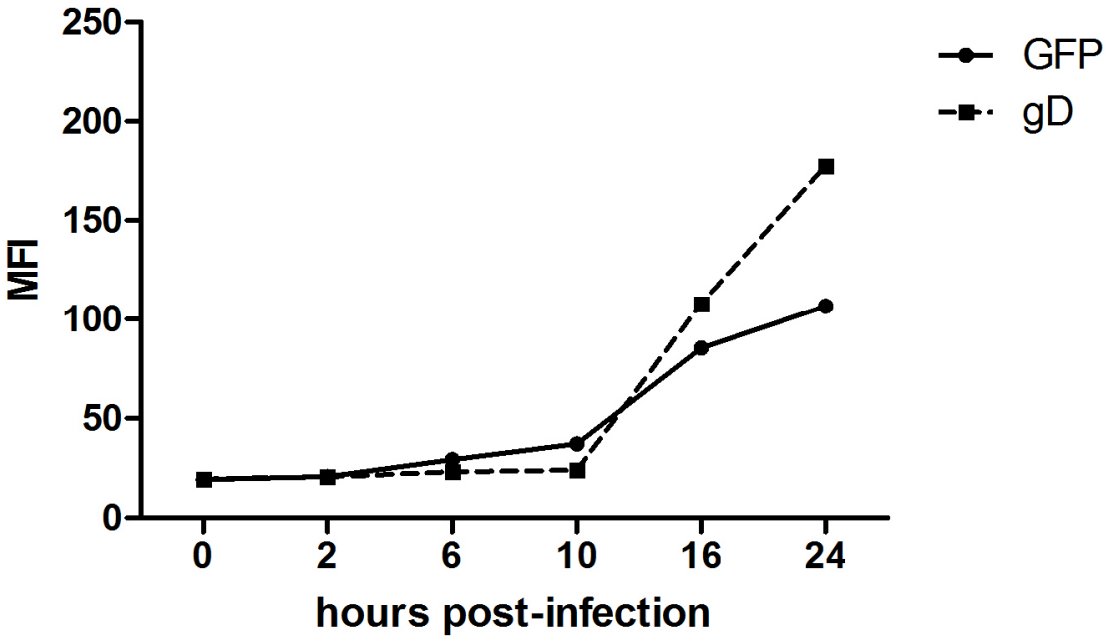
One question that remains unanswered is the nature of the receptor(s) on DC that interact with the glycoproteins to initiate a response. In this thesis this has been explained by examining known HSV-1 receptors, specifically HVEM, nectin-1 as well as TLR2 and TLR9, known to interact with HSV-1 (section 3.2.6). More studies in this

area are necessary to try to establish the exact receptor, perhaps looking at the newly recognized gB receptor, PILRα (Satoh et al., 2008) or the different integrins (Parry et al., 2005). However, these studies will have to take into account the possibility of receptor redundancy, that different receptors may act in concert in order to deliver the activating signal to DC and "receptor switching", where in the absence of one receptor the glycoproteins may interact with another receptor..

Another area of interest is the interaction between HSV-1 infected keratinocytes and DC where a robust in vitro model would be very informative. For example, HaCaT cells are known to express the HSV-1 glycoproteins following infection (appendix A), but neither the crosstalk between the chemokines/cytokines secreted by these cells and DC nor the interaction between the glycoproteins expressed on the HaCaT cells and DC has been studied. In vitro models could also be taken further using skin explants and analyzing the migratory abilities and the activation of different subsets of DC and/or macrophages. In addition the design of appropriate complimentary studies in *in vivo* animal models would be useful.

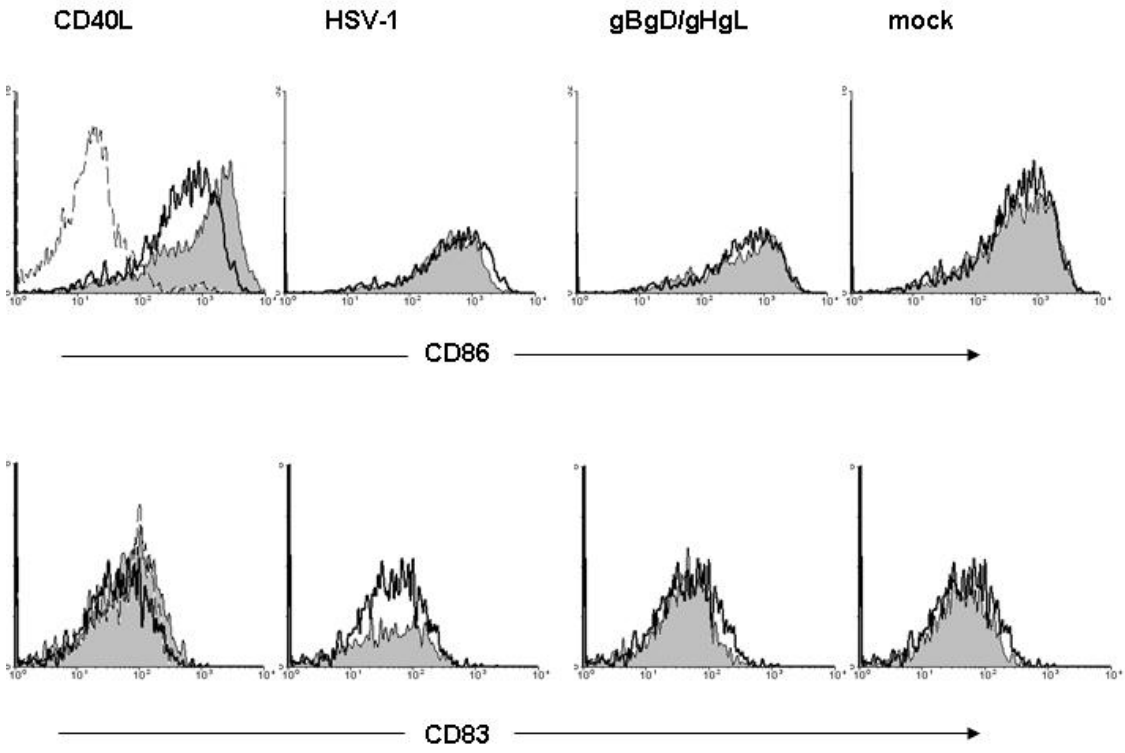
Finally, for use either as a vector for immunotherapy, or as a HSV-1 vaccine, the production of a VLP consisting of the viral glycoproteins should be considered. In preliminary studies, an attempt was made to assemble a VLP from 293T cells expressing the four HSV-1 entry glycoproteins, and carrying the HIV gag protein and a GFP expressing protein. These VLP were found to enter both BHK and 293T cells as confirmed by GFP expression (data not shown), but more studies are necessary to establish and characterize the interaction with DC.

Appendix A – preliminary results



HaCaT cells are highly susceptible to HSV-1 infection showing expression of viral glycoproteins. The keratinocyte cell line, HaCaT, was infected with HSV-1/GFP at an MOI of 1 and the MFI of GFP and gD expression was measured by FACS in a 24 hour time course. The graph represents the mean values from three independent experiments.

Appendix B – preliminary results



***Ex-vivo* isolation of human epidermal LC.** Human skin was obtained from mammoplasty patients with informed consent and local ethical approval. Epidermal and dermal sheets were separated and incubated in complete medium supplemented with GM-CSF. Migratory LC were harvested from the fluid phase. Cells were stimulated and analyzed for CD86 and CD83 maturation markers by flow cytometry. Open dotted histogram in top right represents the isotype control. Open histograms represent the unstimulated LC; grey histograms represent the stimulated cells.

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